THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XVII OCTOBER, 1936 No. 5

STUDIES IN DIPHTHERIA TOXIN PRODUCTION. I: THE EFFECT OF IRON AND COPPER.

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Received for publication July 15th, 1936.

THE effect of small amounts of heavy metal salts on the production of diphtheria toxin has been studied by a number of workers, with conflicting results. Locke and Main (1931) found their veal infusion peptone broth contained between 0·0001 and 0·0004 mg. of iron per c.c. Increasing the iron concentration to 0·006 mg. reduced toxin production from 600 M.L.D. to less than 20 M.L.D. per c.c. Their control media apparently contained more than the optimum iron concentration, and therefore they detected no favourable effect on adding small amounts of iron. The same workers found that there was 0·001 to 0·004 mg. of copper per c.c. in their media, and that further addition of small amounts of copper increased the toxin production slightly. Pope (1932), using a peptone medium, was unable to repeat this latter observation. He found that the addition of 2·0 mg. of iron per litre completely inhibited toxin production. No analyses were reported. Scheff and Scheff (1934), using Wadsworth and Wheeler (1934) "infusion-free" medium, found only 0·00002 mg. of iron per c.c., but apparently did not analyse the sugar and sodium succinate solution added separately to the medium, which undoubtedly contained significant amounts of iron as impurity. Addition of 0·001 mg. of iron per c.c. inhibited toxin production, whereas small amounts of copper exerted a slightly favourable effect. They did not use the flocculation reaction and gave their results as M.L.D. Strøm (1935) examined the effect of adding iron in 10-fold dilutions to Wadsworth and Wheeler's medium. Toxin production was increased from 5 Lf to 9 Lf per c.c. by the addition of 0·001 millimols of iron per litre. Larger amounts were inhibitory. No analyses were reported. Leulier and Sedaillan (1930) found that small quantities of copper exerted slightly stimulating effect on toxin production. A complete review of previous work on the effects of inorganic salts may be found in the monograph by Strøm.

The above *résumé* of the literature shows that all workers agree that iron is inhibitory in relatively low concentration. The exact limits of this inhibitory

action have, however, not been clearly defined, and in only two cases (Locke and Main, 1931; Scheff and Scheff, 1934) are quantitative determinations of iron and copper in the medium available. Moreover, all these experiments have been done on relatively low grade toxin.

We were led to reinvestigate the effect of inorganic salts on toxin production by the accidental observation that the yield of diphtheria toxin produced on infusion-free peptone medium (Wadsworth and Wheeler, 1934) increased by more than 100 p.c. when Fernbach flasks made of soft glass were used instead of the usual Pyrex glass. It was thought that this stimulation of toxin formation might be due to the copper or iron content of the soft glass, and these metals were accordingly studied in detail. In this connection it is of interest that Walbum, as early as 1921, suggested that variation in toxin production from one flask to another might be due to inorganic material extracted from the glass during autoclaving.

EXPERIMENTAL PROCEDURE.

Cultures.—A Park-Williams No. 8 strain of *C. diphtheriæ* obtained from the New York State Laboratories in Albany and labelled No. 5 was used for the greater part of this work. This strain was subcultured daily on infusion-free medium. We have also conducted some experiments with other Park-Williams No. 8 strains from the National Institute of Health (N.I.H. strain), from the Alabama and Michigan State Laboratories, and from the Connaught Laboratory of Toronto.

Media.—Wadsworth and Wheeler (1934) infusion-free Difco Proteose peptone broth and a slight modification of this same medium recommended by Dr. W. E. Bunney, of the Michigan Department of Health, were used throughout most of this work. Both modifications consistently gave 27 ± 2 Lf per c.c. of toxin in the experimental flasks described below with the Albany No. 5 strain.

Except when large quantities of toxin were desired, the organisms were grown in 125 c.c. Pyrex Erlenmeyer flasks. Each flask contained 23 c.c. of medium autoclaved at 10 lb. for 10 minutes. 0·5 c.c. of a solution containing 25 p.c. sodium lactate, 15 p.c. Merck's C.P. maltose and 7·5 p.c. Merck's C.P. dextrose was added separately to each flask before inoculation. With the Michigan medium only the maltose and dextrose were added separately, since sodium lactate and succinate were autoclaved with the peptone in this case. The flasks were inoculated with 24-hour cultures and incubated for 3–4 days at 35° C., after which the flocculation titre was determined. With these small flasks maximum toxin production is attained after 2 to 3 days' incubation.

Analyses.—Iron: Iron was determined with dipyridine according to the method of Hill (1931). Samples of media containing a total of 0·005 to 0·02 mg. of iron were concentrated in Pyrex dishes on the water-bath, transferred to Pyrex Kjeldhal flasks, and ashed by gently boiling to dryness with three 10 c.c. charges of nitric acid which had been re-distilled in a Pyrex still. The ash was dried by gentle flaming and dissolved in 2 c.c. of iron-free distilled water, 0·5 c.c. of a solution containing 63·2 mg. of dipyridine in 100 c.c. of 0·01 *N* HCl was added and the ash made up to 5 c.c. with iron-free sodium acetate solution. The intensity of the pink colour formed after reduction with a little iron-free sodium hydrosulphite was measured in a Zeiss Pulfrich photometer and compared with a standard. Suitable blanks were always made, using the reagents alone.

Copper: Media ashed as above were analysed for copper with sodium diethyldithiocarbamate according to the method of Haddock and Evers (1932). The Pulfrich photometer was used to measure the yellow colour produced. All analyses were made in duplicate.

EFFECT OF GLASS ON TOXIN PRODUCTION.

Infusion-free medium, in our experience, has consistently given 15 to 25 Lf units of toxin per c.c. in Pyrex Fernbach flasks with the Albany No. 5 strain.

For the initial experiment, in which soft glass Fernbach flasks were used, the flasks remained seven days at incubator temperature before inoculation. After 8 days of growth, the toxin when collected and pooled flocculated in 10 minutes and had 43 Lf per c.c. The L+ dose was 0·03 c.c. and the M.L.D. 0·00075 c.c. Individual flasks ran as high as 54 Lf per c.c.

This experiment was repeated under more controlled conditions. Twenty soft glass Fernbach flasks* were inoculated immediately after the preparation of the medium, 4 were inoculated after 7 days and 4 after 10 days at incubator temperature (35° C.). Pyrex flasks containing a quantity of medium to give the same surface-volume ratio served as controls. The flasks were covered with paper caps to prevent evaporation while being kept at 35° C. The results are given in Table I.

TABLE I.—*Toxin Production in Pyrex and Soft Glass Fernbach Flasks.*

Number of flasks.	Type of flask.	Number of days in incubator before inoculation.	Pooled toxin Lf per c.c.
3	Pyrex	3	18·3
20	Soft glass	0	29
4	„	7	41·5
4	„	10	42·5

It was now of interest to find out whether the same effect could be obtained by adding soft glass to media in Pyrex flasks. A soft glass Fernbach flask was powdered in a ball mill and the fraction passing through a 100-mesh sieve but held back by a 200-mesh sieve was added in diminishing quantities to infusion-free medium in 125 c.c. Pyrex Erlenmeyer flasks before autoclaving. Table II gives the results of two such experiments.

TABLE II.—*Effect of Powdered Soft Glass on Infusion-free Medium in Pyrex Erlenmeyer Flasks.*

Mg. of powdered glass added to 25 c.c. of medium.	Toxin produced in Lf per c.c. after 3 days at 35° C.	
	Experiment 1.	Experiment 2.
0	27	27
0·15	28·5	29
0·31	31	31
0·63	27	27
1·25	24	18
2·50	...	15
5	22·5	14
10	15	19·5
20	11	9
100	Poor growth	Poor growth
200	No growth	No growth

* These soft glass Fernbach flasks were made of the so-called " Perfection " glass by the Kimble Glass Company in 1924.

It will be noted that the addition of only 0·3 mg. of powdered glass exerts a small but definite stimulating effect on toxin production, whereas larger amounts are definitely inhibitory. It seemed quite clear from this experiment that the greater toxin production in soft glass as compared with Pyrex flasks is due to a stimulating effect obtained from the soft glass, not to an inhibiting effect of Pyrex glass. However, since Pyrex glass is known to contain considerable amounts of boron, this element was tested for a possible inhibitory effect. Sodium borate when present in infusion-free medium in concentrations varying from 10^{-3} to 10^{-9} molar had no effect whatever on the amount of toxin produced.

THE EFFECT OF IRON ON TOXIN PRODUCTION.

In our search for the element responsible for the stimulatory action of soft glass we first studied the effect of addition of iron salts to infusion-free medium. Although we have been unable to detect any appreciable increase in the iron content of media autoclaved with powdered glass, the effect of adding ferric chloride closely paralleled that of powdered soft glass, and the results obtained have proved of great significance in the present work.

In Fig. 1 are plotted the results of three separate experiments on the effect of adding iron to infusion-free medium. The Lf per c.c. value is plotted as ordinate against the milligrams of iron present in 25 c.c. of medium. It will be noted that the curve rises steeply to a sharp maximum of 31 Lf per c.c. when 0·0036 mg. of iron are present. As little as four times this quantity of iron is sufficient to inhibit toxin formation completely. A small change in iron content, therefore, causes a relatively large change in toxin produced.

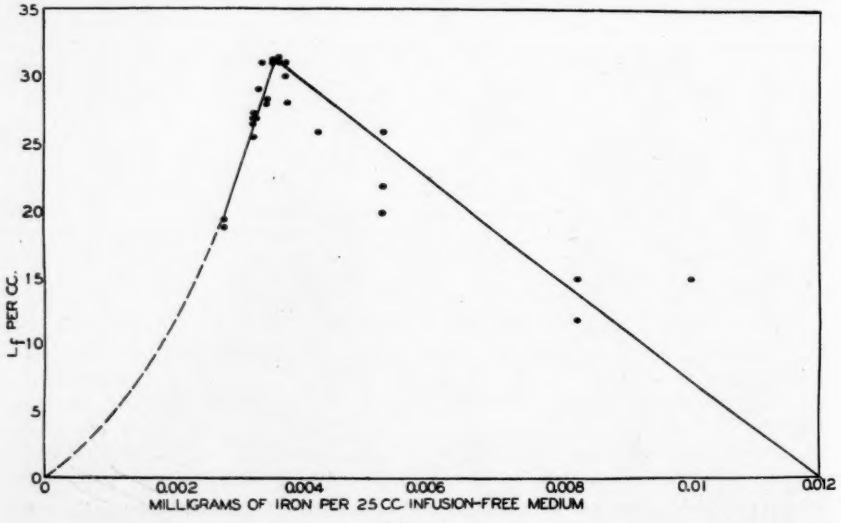

FIG. 1.

Analyses of three separate batches of infusion-free medium (Michigan formula) without addition of the sugars showed the presence of 0·00012± 0·00002 mg. of iron per c.c. Therefore each flask contained 0·00275 mg. of iron. Analyses of the sugars showed that the Merck's C.P. maltose used contained 0·0052 mg. iron per g. and the C.P. dextrose 0·001 mg. per g. Addition of 0·3 p.c. maltose and 0·15 p.c. dextrose, therefore, brought the total iron content of the control flasks to 0·0032 mg. This amount of iron is very close to the optimum iron concentration of 0·0036 mg. per 25 c.c. It was found that by boiling the sugar solution with calcium chloride and disodium phosphate, the iron which it contained could be completely removed with the precipitated calcium phosphate. When such iron-free sugar solutions were used, the toxin production dropped from 27 Lf per c.c. in the controls to 19 Lf per c.c. It seems probable that if the remaining iron could be removed from the medium, no toxin would be formed. The broken-line curve in Fig. 1 represents this prediction. The presence of 0·0005 p.c. iron in C.P. maltose affords an explanation of the fact noticed by Wadsworth and Wheeler that technical maltose may be superior to more highly purified preparations for producing diphtheria toxin.

In the preparation of infusion-free media Wadsworth and Wheeler emphasize the importance of heating the calcium and phosphate ions together in the presence of the peptone in alkaline solution in order to obtain potent toxin. Their results were particularly striking when Parke Davis peptone decalcified with ammonium oxalate was used. It seemed probable to us that this curious phenomenon might be explained by the removal of excess iron from the medium. Table III shows that this is indeed the case. Media made with Parke Davis peptone treated with ammonium oxalate in order to remove as much calcium as possible contained 0·003 mg. of iron per c.c.—an amount considerably in excess of that necessary to prevent all toxin formation. Boiling with calcium chloride in alkaline solution removed this excess iron.

TABLE III.—*Toxin Production with "Decalcified" Parke Davis Peptone.*

Medium.	Iron content mg. per c.c.	Toxin produced after 3 days at 35° C. Lf per c.c.
Decalcified Parke Davis peptone	0·003	0
Parke Davis peptone boiled with $CaCl_2$ and K_2HPO_4	$<0·0003$	19

ADDITION OF "BOUND" IRON TO INFUSION-FREE MEDIUM.

A few experiments have been conducted on the effect of adding iron in the form of hæmin. Re-crystallized hæmin in very low concentration was found to stimulate toxin production. In some cases the addition of only 0·00015 mg. of hæmin to 25 c.c. of infusion-free medium sufficed to increase the toxin formed from 28·5 Lf to 39 Lf per c.c. Complete inhibition of toxin production did not occur until more than 0·6 mg. of hæmin had been added; but this may have been due to traces of inorganic iron present as impurity. One experiment with crystalline hæmoglobin, added in equivalent quantity, raised the Lf per c.c. from 28 to 36.

THE EFFECT OF COPPER IN TOXIN PRODUCTION.

Analysis showed 0·022 mg. of copper per 25 c.c. of infusion-free medium. Further addition of copper as copper sulphate produced no effect until a quantity large enough to diminish growth had been added. A typical experiment is given in Table IV.

TABLE IV.—*Effect of Copper on Toxin Production in Infusion-free Medium.*

Mg. of $CuSO_4.5H_2O$ added to 25 c.c. of medium.	Toxin produced in Lf per c.c. after 4 days at 35° C.	Growth.
0	24	Good
0·05	24	,,
0·1	22	,,
0·2	23	,,
0·4	23	,,
0·8	21	,,
1·6	12	Fair
3·1	0	None

On autoclaving 20 mg. of powdered glass with 25 c.c. of infusion-free medium, the copper content was increased from 0·022 to 0·057 mg. in 25 c.c.; 200 mg. of powdered glass inhibits growth entirely, and this amount of glass would thus be expected to liberate 0·35 mg. of copper. This figure is in fairly good agreement with the amount of copper, 0·78 mg., necessary to prevent growth. Thus, although sufficient copper was liberated from the soft glass used to account for the inhibitory effect on growth, we were unable to find any evidence that this element was responsible for the increased yield of toxin obtained when the organisms were grown in the soft glass flasks.

The quantitative determinations of iron and copper which we have reported are in good agreement with the figures given by Locke and Main (1931) and Scheff and Scheff (1934) for the iron and copper contents of veal infusion broth and infusion-free medium.

DISCUSSION.

It is clear that the concentration of inorganic salts, even in minute traces, and particularly of iron salts, must be carefully controlled in order to produce potent diphtheria toxin. Slight changes in the concentration of the heavy metal salts present may cause relatively large fluctuations in the amount of toxin produced. We believe that the variation in toxin production from flask to flask, from one batch of medium to another, and the frequent inability of one laboratory to produce toxin on media recommended by others, may often be attributed to this cause. For example, this laboratory has for some months attempted, without success, to produce diphtheria toxin on the pig stomach digest medium recommended by Taylor (1935). This medium, which consistently yields toxins of more than 70 Lf per c.c. at the Connaught Laboratories in Toronto, has failed to produce any toxin whatsoever when prepared in this laboratory. Analysis with dipyridine has shown that more than 0·001 mg. of iron is present per c.c. in the medium prepared by us, whereas in media

kindly sent us by Miss Taylor no iron was detected with this reagent and toxin with an Lf value of 72 per c.c. was produced. The reason for this difference in iron content has not been determined. On removing the excess iron from our medium by forming a precipitate of calcium phosphate in alkaline solution, we have obtained toxin of 21 Lf per c.c. Although this figure admittedly falls far short of that given by the Toronto workers, it should be noted that this was a " re-worked ", and, therefore, possibly impaired medium. At any rate, the marked increase in toxin production following the calcium phosphate treatment illustrates the trifling difference between success and failure in toxin production.

The amount of iron necessary to prevent diphtheria toxin formation is considerably less than the amount found in normal tissues. It is, therefore, significant that all methods for preparing toxin media involve the removal of a flocculent precipitate formed in alkaline solution. Excess iron is presumably removed at this point. In our experience all media giving a visible colour test for iron with dipyridine have failed to produce any diphtheria toxin.

It has often been claimed that no relationship exists between growth and toxin production. This is obviously the case if, for example, the quantity of iron present is allowed to exceed the concentration necessary for optimum toxin production. It is possible, however, that if the inorganic salt concentration be suitably controlled, a relationship may be found.

SUMMARY.

1. Sufficient inorganic material may be dissolved out of the glass vessels used in preparing diphtheria toxin to alter the amount of toxin produced by more than 100 p.c.

2. The effect of iron and copper salts on toxin production in infusion-free peptone medium has been studied. The optimum concentration of iron in this medium, for the strain used, was 0·00014 mg. per c.c., while 0·0005 mg. per c.c. inhibited toxin production altogether. No visible effect on growth was observed between the optimum and 0·06 mg. of iron per c.c. In our experience all media giving a visible colour test for iron with dipyridine have failed to produce diphtheria toxin.

3. The infusion-free medium prepared in this laboratory contained 0·00086 mg. of copper per c.c. Further addition of copper had no effect on the toxin produced until added in quantity sufficient to impair growth.

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STUDIES IN DIPHTHERIA TOXIN PRODUCTION. II: PRODUCTION OF POTENT DIPHTHERIA TOXIN ON A SIMPLE AMINO-ACID MEDIUM.

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Received for publication July 15th, 1936.

ANY attempt to isolate diphtheria toxin in a state approximating purity has seemed to us futile so long as the usual complex media are employed. The recent success of Mueller and his associates (1935) in obtaining good growth of toxigenic strains of diphtheria bacilli on media consisting of amino-acids, salts and a small quantity of an as yet unknown but highly purified fraction obtained from liver extract seemed to provide a suitable starting-point from which to work. A complete review of the numerous attempts to produce toxin on " synthetic " media may be found in the monograph by Strøm (1935). Few workers have succeeded in getting good growth of toxigenic strains on simplified media, and in no case have potent toxins been produced. Very weak toxin production on synthetic media has been reported by Uschinsky (1897), Hadley and Gorham (1907), Braun *et al.* (1929), Wadsworth and Wheeler (1934), Maver (1931) and Schmidt (1933). In no case was sufficient toxin obtained to give the flocculation reaction or an M.L.D. below 0·06 c.c. Hosoya, Ozawa and Tanaka (1933) have, however, recently obtained toxin of 5 Lf and 200 M.L.D. per c.c. on a sulphuric acid hydrolysate of a methyl alcohol extract of peptone.

Our early attempts to produce toxin on Mueller's medium met with complete failure. Addition of sugars, organic acids, tissue extracts and finally of peptone itself were of no avail, and we were forced to give up this line of work for the time being. However, as soon as it was realized that minute amounts of iron are sufficient to completely inhibit toxin formation (Pappenheimer and Johnson, 1936), we again returned to a study of Mueller's medium. A test with dipyridine revealed the presence of a large excess of inorganic iron, most of which was contained in the fraction from liver extract. After the medium had been boiled with calcium chloride this excess of iron was carried down with precipitated calcium phosphate and potent toxin was produced. Although the optimum concentrations of iron and copper have not been worked out as yet, media prepared in the following way give 20 Lf per c.c. of toxin with the Albany No. 5 strain.

The following substances are weighed out and dissolved in 500 c.c. of distilled water : *l*-cystine 0·2 g., *dl*-glycine 0·5 g., *dl*-valine 1·0 g., *dl*-methionine 0·2 g.,

dl-alanine 0·2 g., *l*-tyrosine 0·1 g., *dl*-leucine 0·5 g., *d*-glutamic acid hydrochloride 5·0 g., sodium chloride 5·0 g., dipotassium phosphate 5·0 g., liver extract preparation* 25 c.c.

The pH is adjusted with 5*N* NaOH to 7·8 and 300 mg. of calcium chloride added. After boiling for 30 minutes, the solution is filtered and 300 mg. of magnesium chloride and 100 mg. of *l*-tryptophan are added. The solution now gives no colour on testing for iron with dipyridine. Finally the solution is made up to 1000 c.c. with distilled water, readjusted to pH 7·8 and distributed in 125 c.c. Pyrex Erlenmeyer flasks, 24 c.c. to each flask, and autoclaved at 10 lb. pressure for 10 minutes. Before inoculation 0·5 c.c. of a sterile solution containing 25 p.c. sodium lactate, 15 p.c. Merck's C.P. maltose and 7·5 p.c. Merck's C.P. dextrose is added to each flask.

After three days' incubation on this medium the Albany No. 5 strain gave 20 Lf per c.c. of toxin with an L+ dose of 0·1 c.c. and M.L.D. of 0·002 c.c. Table I shows the amount of toxin produced by five Park-Williams No. 8 strains in a similar medium, in which the magnesium chloride was added before removing the calcium phosphate precipitate, and which contained 100 mg. of *d*-histidine hydrochloride per litre. All five strains gave excellent growth on this medium.†

TABLE I.—*Toxin Produced by Different Strains of* C. diphtheriæ *on Amino-Acid Medium.*

Strain.	Lf per c.c.	L+ dose.	M.L.D.
Albany No. 5	15	0·15 c.c.	0·003 c.c.
Albany No. 5	18	0·10 ,,	$>0{\cdot}001 <0{\cdot}005$ c.c.
Toronto	12	0·10 ,,	0·003 c.c.
Alabama	9	—	—
Michigan	9	—	—
N.I.H.	6	—	—

DISCUSSION.

It has been generally accepted by most workers in the field that peptone is essential for the production of potent diphtheria toxin. It has been assumed that higher polypeptides and albuminoses are the active substances present in the peptone. Since potent toxin has now been produced on a simple amino-acid medium, plus an accessory growth factor, one is in a more favourable position to isolate diphtheria toxin and investigate its nature.

* We are indebted to Dr. J. Howard Mueller of the Harvard Medical School for this liver extract preparation. It contained material from an aqueous extract of liver, soluble in 95 p.c. alcohol, adsorbed on wood charcoal and eluted with acid alcohol. The active material in this preparation was small in comparison with the amounts of amino-acids used and should be regarded as an accessory growth factor.

† It should be emphasized that some Park-Williams No. 8 strains might grow poorly on the medium described. Dr. Mueller informs us that of ten supposedly Park-Williams No. 8 strains of *C. diphtheriæ* tested by him, no two have exactly the same nutritional requirements. We feel confident, however, that the above medium will give growth and toxin production with the majority of strains.

SUMMARY.

By taking steps to control the iron concentration, potent diphtheria toxin of 20 Lf per c.c. and an M.L.D. of 0·002 c.c. has been prepared on a simple amino-acid medium.

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THE INTERPRETATION OF GROWTH FLUCTUATIONS IN TRANSPLANTABLE TUMOURS.

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Received for publication June 24th, 1936.

FLUCTUATIONS in the rate of growth and in the proportion of successful inoculations are a constant feature in many transplantable tumours. Mouse tumours 206, an alveolar mammary carcinoma, and 2529, a polymorphous sarcoma, provide convenient material for the study of this phenomenon, for they both have well-defined fluctuations over a relatively short period of time. They both regress frequently and belong to the group of tumours which produce concomitant immunity (Russel, 1912).

Tumours 206 and 2529 were transplanted at fortnightly intervals by inoculating ten mice subcutaneously with a small graft through a hollow needle. The grafts were prepared from the most progressive tumour of the previous passage. The tumours were charted weekly as silhouettes on squared paper

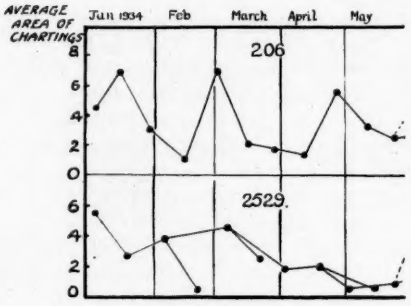

FIG. 1.

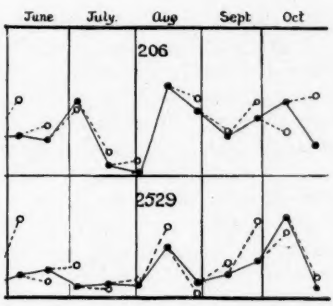

FIG. 2.

FIG. 1.—Growth fluctuations of tumours 206 and 2529 over a period of five months. The fluctuations in the growth of tumour 206 are larger and more frequent than those in the growth of tumour 2529.

FIG. 2.—Growth fluctuations of tumours 206 and 2529 during their simultaneous propagation in the same mice over a period of five months, represented by the continuous line. The average amounts of growth in the control single inoculations are represented by circles. Except for the third passage there is a general correspondence between the two curves. The fluctuations in growth are therefore largely determined by variations in the resistance of the mice.

The mice used for the propagation of tumours 206 and 2529 during the period covered by Figs. 1 and 2 were of mixed stock, and were all supplied by the same animal dealer.

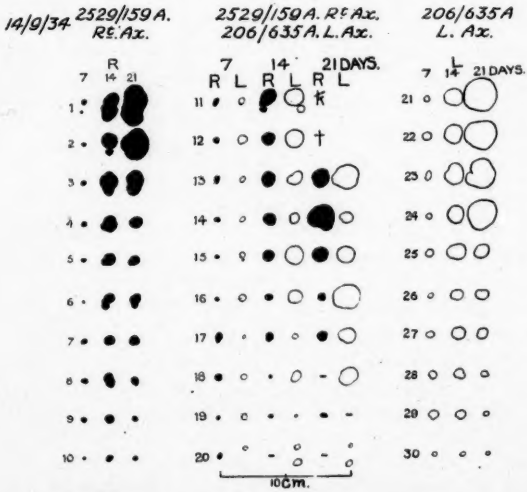

FIG. 3.—Mice 1–10 inoculated in the right flank with 0·02 g. of tumour 2529. Mice 11–20 inoculated in the right flank with 0·02 g. of tumour 2529 and in the left flank with 0·02 g. of tumour 206. Mice 21–30 inoculated in the left flank with 0·02 g. of tumour 206. The tumours 2529 and 206 which supplied the grafts were obtained from the right and left flank respectively of a mouse in the previous double transplantation. This chart represents the eighth passage of the simultaneous propagation (Fig. 2). The tumours tend to grow similarly when transplanted into the same mice. As indicated by the control single inoculations, the tumours have little effect on each other when grown simultaneously.

(5 squares = 1 sq. cm.). By counting the squares included in the silhouettes at the second charting and dividing by the number of animals charted, the average amount of growth at two weeks may be calculated. In Fig. 1 the ordinates show this for consecutive transplantations of tumours 206 and 2529 during a period of five months. Although this method of estimating is by no means accurate for individual experiments, it gives a good representation of the fluctuations in growth. It will be seen that during the period in question the fluctuations in the growth of tumour 206 were larger and more frequent than of tumour 2529. This would suggest that the type of fluctuation is characteristic of the tumour, and that it is determined by cyclical changes in the tumour parenchyma.

The two tumours were next propagated simultaneously in the same mice through eleven successive passages, 206 in the left flank and 2529 in the right flank. Each double transplantation was made into ten mice from two tumours growing in the same mouse. As a control to the effect of one tumour on the growth of the other, ten mice were inoculated singly from either tumour as shown in Fig. 3, which is the chart of the eighth passage in this series. The tumours tend to grow similarly when transplanted into the same host. This is frequent but not general, as is the case in simultaneous inoculations of the same tumour. The control single inoculations indicate that the tumours have little effect on each other when grown simultaneously. In Fig. 2 the fluctuations in growth of the two tumours during their simultaneous propagation are represented, along with the average amount of growth for each control single inoculation. Again the fluctuations of the two tumours are pronounced, but there is a general correspondence in the two curves, except for the third passage. The conclusion appears justified that the fluctuations in growth in mouse tumours 206 and 2529 are largely determined by variations in the natural resistance of the mice.

DISCUSSION.

The relative importance of the graft and the host in producing fluctuations in transplantable tumours has been studied by many workers. Bashford, Murray and Cramer (1905), after a careful analysis of the fluctuations in the Jensen mouse carcinoma, came to the conclusion that "the power of establishing themselves in new hosts varies periodically in these tumour-cells from inherent causes". This conclusion was further affirmed by Bashford, Murray, Haaland and Bowen (1908) from a study of the fluctuations in four different tumour strains. On the other hand, according to the genetic theory of transplantation, as formulated by Strong and Little (1924), "the fate of the implanted tumour tissue when placed in a given individual (host) is brought about by a reaction between the host, determined to a large extent by its genetic constitution, and the transplanted tumour-cell, controlled to some extent by its genetic constitution". Bittner (1932), in a criticism of the work of Bashford, Murray and Cramer, has reproduced one of their curves by inoculating a tumour into groups of 10 mice composed of varying proportions of two pure strains of mice, one of which was wholly susceptible and the other wholly resistant to the tumour employed. From this result he maintains that rhythms

of growth do not occur in the transplanted tumour-cell, although he admits the criticism that Bashford's data were secured over a period of 500 days, whereas his own results were for 2 days.

The similarity of the behaviour of tumours 206 and 2529 when they are propagated simultaneously in the same mice suggests that an additional factor, besides differences between the genetic constitution of the host and the respective tumours, is involved, namely, variations in the mice in respect of their natural resistance to transplantation in general. In another publication by the present author (Selbie, 1936) it has been shown that this factor is hereditary, for, by selective breeding of rats for susceptibility to the Jensen rat sarcoma, a strain of rats can be obtained which is more susceptible to other rat tumours. The question whether periodic variations occur in the tumour-cells themselves can only be settled by propagating tumours in animals of uniform genetic constitution.

Tumours 206 and 2529, by virtue of their great ability to call forth the resistance of the host, show differences in the receptivity of the mice which are not shown by the more progressive tumours. In experiments with transplantable tumours it is therefore necessary to investigate the natural resistance of the stock of animals to be used. Particularly in therapeutic experiments, the tumour employed should be able to overcome or be indifferent to the natural resistance of the animals, and, if possible, a pure strain of animals should be used.

SUMMARY.

Mouse carcinoma 206 and mouse sarcoma 2529, when grown simultaneously in the same mice, show similar growth fluctuations.

The fate of a tumour-graft depends to a great extent on the susceptibility of the animal to transplantation.

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SUSCEPTIBILITY TO TUMOUR GROWTH IN RATS.

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Received for publication June 24th, 1936.

CHAMBERS and Scott (1930) have shown that the receptivity of a strain of rats to the Jensen rat sarcoma can be altered by selective breeding. Owing to unsatisfactory results with rats from various animal dealers they began breeding from their own stock. Many of the animals used for breeding had been inoculated with the Jensen rat sarcoma and had had their tumours removed by operation, while others had reabsorbed their tumours. This practice was continued for three years and, during that time, the number of animals with no tumour twenty days after inoculation was 6 p.c., whilst those with tumours of less than 3·5 c.c. which eventually disappeared was about 14·5 p.c. The practice of operating on tumour-bearing rats was then suspended and many rats that had reabsorbed their tumours were used for breeding. After two years the number having no tumours at twenty days had increased to 54·5 p.c. and, if the disappearing tumours were included, to 66 p.c. Chambers and Scott concluded that this result was due to continued breeding from the more immune stock.

Before 1932 many of the laboratory stock rats of the Imperial Cancer Research Fund had been bred from rats in which a tumour had failed to grow or had been reabsorbed. The results of inoculation with the Jensen rat sarcoma were very unsatisfactory. From November, 1931, to April, 1932, 65 laboratory stock rats were inoculated by the needle technique, and of these, 54 p.c. did not develop tumours or reabsorbed them. In order to improve the stock the technique of Chambers and Scott was followed. Rats bearing large, progressive tumours had their tumours removed by operation, usually at the 17th day after inoculation, and were kept for breeding. The first litter from them was available in May, 1932, and from that time the Jensen rat sarcoma has been continuously propagated in rats whose parents have had large, progressive tumours.

It was almost immediately evident that this practice was worth the trouble, for during the first three months the number of progressive tumours among 18 inoculated rats was 62 p.c. as against 46 p.c. in the previous six months. The number of rats inoculated and the proportion of progressive tumours for each six-month period up to October, 1935, are given in Fig. 1. Animals dying before the 17th day after inoculation are not included. After the first six months the percentage of progressive tumours in the respective periods has never dropped below 72, and during the last year of the experiment has been over 93. In fact, during the last year there were only 4 non-progressive tumours in 132 inoculated rats.

At various times a batch of the original laboratory rats was inoculated with the Jensen rat sarcoma as a control to the selected strain. In every experiment the number of progressive tumours was greater in the selected rats ; a typical experiment is shown in Fig. 2. This was true even when the proportion of progressive tumours in the selected strain was low as in the period November, 1933, to April, 1934, when, in the series 427E (inoculated November 23rd, 1933), the selected strain yielded 7 progressive tumours out of 12 inoculations, and the unselected strain only 1 out of 12.

Towards the end of 1935 the receptivity of the selected and unselected rats to the Jensen rat sarcoma and to other rat tumours was compared. All the inoculated rats were re-inoculated with the same tumour after an interval of 10 days. The results are shown in Fig. 2. As was expected, there is in the case of the Jensen rat sarcoma a great difference in receptivity. The unselected rats gave extremely variable results, whereas the selected rats were

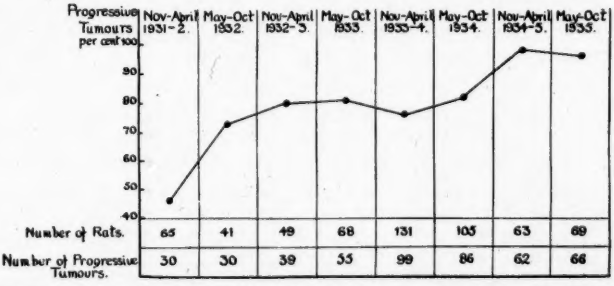

FIG. 1.—The total number of rats inoculated with the Jensen rat sarcoma, and the number and percentage of progressive tumours during each six-month period from November, 1931, to October, 1935. The propagation of the Jensen rat sarcoma in selected rats was begun in May, 1932. There is an immediate rise in the proportion of progressive tumours, which has been maintained and further increased during the succeeding two years.

almost uniformly susceptible. The Guérin (1934) rat carcinoma, the Willis rat carcinoma and the Walker rat carcinoma 256 also grew more progressively and more uniformly in the selected strain. Rat sarcoma 46 grew uniformly in this strain, whereas in the unselected rats it progressed in three and was reabsorbed in three.

Reinoculation of the Jensen rat sarcoma gave five out of six positive results in the selected and only two out of six in the unselected rats. Reinoculation of the Guérin rat carcinoma gave two positive results in the four surviving selected rats and only one positive in the unselected rats. Positive results on reinoculation were obtained with rat sarcoma 46 only where the primary inoculations gave rise to more progressive tumours ; thus there is only one positive result in the selected and three in the unselected rats. The Willis carcinoma, on reinoculation, gave five positive results out of six in the selected rats and three out of the four surviving unselected rats. Here there were two positive reinoculations after negative primary inoculations in two rats, as was the case with one unselected rat in the Guérin rat carcinoma series. In these the primary inoculations were probably not sufficiently

vigorous to call forth an effective concomitant immunity. On reinoculation the Walker rat carcinoma gave five out of six positive results in the selected

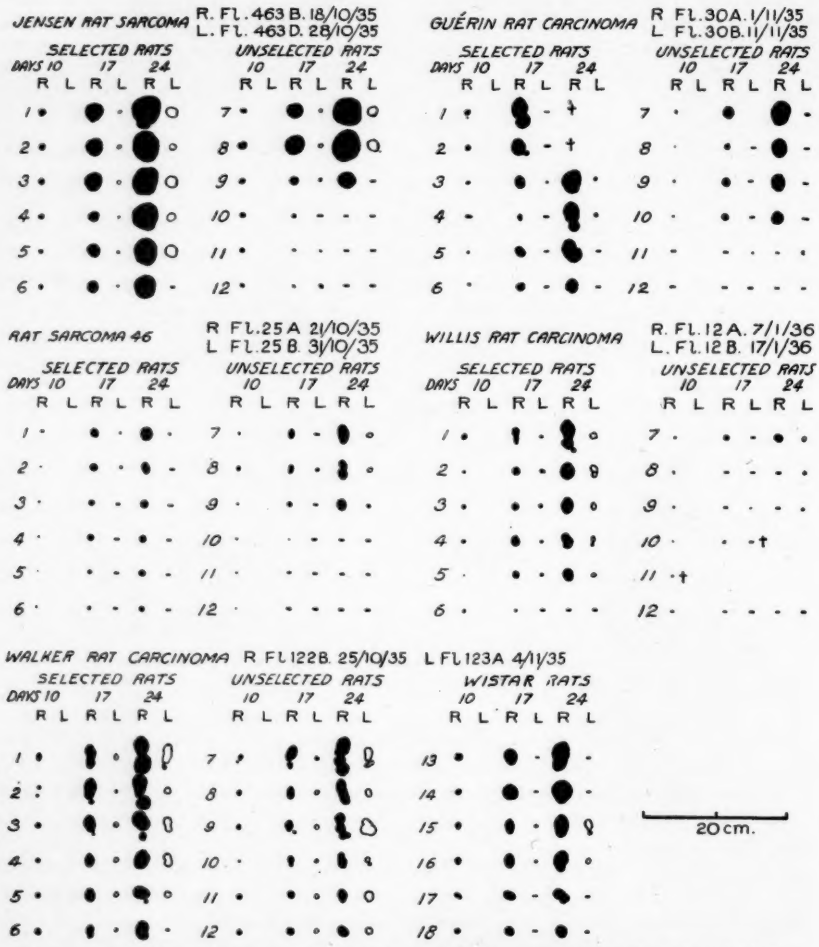

FIG. 2.—Comparison of the growth of the Jensen rat sarcoma and four other transplantable rat tumours in selected and unselected rats. The rats were inoculated with 0·02 g. of the appropriate tumour in the right flank by the needle method, and after an interval of 10 days were re-inoculated in the left flank with the same tumour. In the case of the Walker rat carcinoma, six Wistar rats are included in order to show the ability to produce concomitant immunity in the presence of progressive primary tumours.

rats and six out of six in the unselected rats. On the other hand, in Wistar rats, only two out of six were positive on reinoculation, although the primary tumours were as progressive in growth as in the two laboratory strains.

DISCUSSION.

The results described above suggest that two factors are involved. The greater uniformity with which five different rat tumours have been propagated in the selected strain of rats can be accounted for by the greater homogeneity of the strain resulting from selective breeding. On the other hand, the increased susceptibility of this strain to all but one of the rat tumours suggests that it is more susceptible to transplantation in general.

It may be concluded that uniformity in the results of transplantation can be obtained only in pure strains of animals. The more susceptible a tumour is to the resistance of the host, the more irregular will be the results of transplantation in animals of mixed stock.

The results of reinoculation also demonstrate the greater susceptibility of the selected rats to the Jensen rat sarcoma and perhaps also to the Guérin rat carcinoma, though, as Fig. 2 shows, the experiment with the Guérin tumour was vitiated by the death of two animals. From the results of reinoculation of the five tumours in the two laboratory strains of rats, it might be concluded that reinoculation is more likely to be successful where the primary tumour is more progressive. However, in the Wistar rats inoculated with the Walker rat carcinoma only two reinoculations were positive in six rats, all of which bore progressive tumours. These rats, though they are as susceptible to the growth of the tumour as the laboratory rats, differ in their ability to become immune. The power of a tumour to induce concomitant immunity may therefore vary in different strains of animals. Foulds (1930) has shown that it may vary in different substrains of one tumour and at different times in the same tumour. These complications must be eliminated in every therapeutic experiment with a transplantable tumour by determining at the time of the experiment the behaviour of the tumour in the animals used.

SUMMARY.

A strain of rats susceptible to the growth of the Jensen rat sarcoma has been bred from a relatively insusceptible stock by continuous selection of rats that have had large progressive tumours removed by operation.

This strain is more susceptible to the growth of three out of four other rat tumour strains and gives more uniform results with all four.

A transplantable tumour may produce a greater degree of concomitant immunity in one strain of rats than in another.

Consistent results in experiments with transplantable tumours can be obtained only by using pure strains of animals.

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STUDIES ON NEUROTROPIC RIFT VALLEY FEVER VIRUS THE SUSCEPTIBILITY OF RODENTS.

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Received for publication July 2nd, 1936.

In a preliminary communication (Mackenzie and Findlay, 1936) a method was described for the production of a neurotropic variant of the virus of Rift Valley fever. In the present communication this description is amplified, and such experimental details as were omitted from the earlier report are supplied. The virus of Rift Valley fever first described by Daubney, Hudson and Garnham (1931) has now been maintained in this laboratory for nearly six years, generally by subcutaneous or intraperitoneal passage from mouse to mouse. It has, however, occasionally been inoculated into sheep, goats and other animals (Findlay, 1931–32), and has sometimes accidentally infected man. Throughout all these transfers, and the numerous experimental procedures to which it has been subjected, the characters of the virus have remained unaltered.

In working with Rift Valley fever virus it is impossible not to be struck by the close resemblance that exists between its behaviour and properties and those of yellow fever virus, although these two viruses are quite immunologically distinct.

Since Theiler (1930) had shown that the virus of yellow fever could, under certain conditions, be converted into a neurotropic fixed variant, it was naturally thought that under somewhat similar experimental conditions a neurotropic form of the Rift Valley virus might also be produced. Unfortunately, although simple in theory, the production of this variant form was not easy in practice. Theiler, in his work on yellow fever, made use of the white mouse as the experimental animal. In this species, which is completely resistant to the active agent of yellow fever when injected intraperitoneally, the virus, when inoculated from mouse brain to mouse brain, was so modified that it no longer produced viscerotropic lesions in rhesus monkeys or other susceptible animals. It nevertheless retained the power of rendering them immune against infection with the viscerotropic strain of the virus.

In the case of Rift Valley fever, the mouse did not at first appear to be a suitable animal, owing to its great susceptibility to the disease, and because, when inoculated intracerebrally, mice died in the ordinary way with the characteristic liver necrosis. Of the other common laboratory animals, some, such as the rabbit and guinea-pig, were insusceptible even when inoculated intracerebrally, and the virus could only occasionally be recovered from

them, while others, such as the sheep, goat and monkey, were nearly as susceptible as white mice to intraperitoneal inoculation.

Recently Findlay and Stern (1935) demonstrated the "essential neurotropism" of the yellow fever virus. In their experiments, rhesus monkeys were passively immunized against yellow fever by intraperitoneal inoculation of yellow fever immune serum, and then inoculated intracerebrally with the ordinary viscerotropic, or rather pantropic, strain of the virus. A similar technique has been independently employed by Penna (1936). As a result of the protective action of the immune serum on the viscera, the animals no longer died with liver lesions, but as the central nervous system was not immunized at the same time, the serum failed to protect the monkeys against the neurotropic action of the virus, and, in due course, encephalitis developed. A similar experimental technique would, it was hoped, make it possible to demonstrate for the Rift Valley fever virus also a neurotropic activity, and, if such existed, it seemed not unreasonable to suppose that continued brain-to-brain passage in susceptible, but passively immunized animals, would result in the production of a fixed neurotropic strain. With these objects in view the following experimental work was carried out.

PRODUCTION OF THE FIRST NEUROTROPIC STRAIN.

As a matter of convenience it was decided to perform the first experiments on mice. Having little or no guide as to details of procedure, the mice were inoculated intracerebrelly with 0·03 c.c. of a suspension of infected mouse blood in saline about 15 minutes after they had been given an intraperitoneal injection of immune serum. After two days the mice were killed, their brains removed, ground up in saline, and inoculated into a fresh batch of immunized mice, and so on.

Unfortunately, at the time the experiments were begun an adequate supply of immune serum was not at hand, and thus the amount of serum injected varied from time to time according to the amount available. However, in spite of the fortuitous planning of the experiment a change in the activity of the virus gradually became apparent, and by the time the 10th passage had been reached, a strong neurotropic activity had developed, with a corresponding diminution of the viscerotropic action. This, the first neurotropic strain, has been passaged over 40 times in passively immunized animals, and also in numerous non-immune mice, and may now be considered a partially fixed variant.

As it would not be satisfactory to describe in detail the improvised technique employed in the production of this first neurotropic strain, it was decided that the creation of a second strain should be attempted under more standardized conditions. If this should be successful, it would serve to show that the production of the original strain was not merely fortuitous.

PRODUCTION OF A SECOND NEUROTROPIC STRAIN.

The experience which had been gained from a study of the production of the first strain indicated that sufficient immune serum should be used in order

to prevent, if possible, any specific action of the Rift Valley virus on the liver. For this purpose 6 mice were injected intraperitoneally with 0·5 c.c. of immune human serum obtained from a laboratory assistant who had contracted the disease about a year previously. Fifteen to 30 minutes later the mice were anæsthetized and inoculated intracerebrally with 0·03 c.c. of an active suspension of virus, consisting of infected mouse blood.

On the 5th or 6th day following the inoculation all these mice were either dead or showing certain characteristic symptoms, such as turning movements, paralysis of the limbs, or convulsions, all suggestive of involvement of the central nervous system. One of the mice was then killed and the brain removed, ground up in 5 c.c. of normal saline solution and lightly centrifuged. A fresh batch of mice immunized as before were then inoculated intracerebrally with 0·03 c.c. of the supernatant fluid, suitable cultures being made as controls of sterility. At the same time another batch of normal non-immunized mice were given an intraperitoneal injection of the brain suspension, in order that any change in the viscerotropic activity of the virus might be noted.

From the first it was found that the amount of immune serum given was not always sufficient to protect the mice against the viscerotropic action of the virus, and that the liver tissue often showed small areas of focal necrosis. Since it was not feasible to increase very much the amount of immune serum used, it was decided that the above procedure should be repeated in the hope that the viscerotropic activity of the virus would be suppressed in time.

It has already been mentioned that symptoms in the first batch of mice did not develop until the 5th or 6th day after inoculation. In some of the later passages symptoms were well established in 4 days, and from the 9th passage onwards they often appeared on the 3rd day. There was thus a gradual shortening of the incubation period.

Of the non-immune mice which were inoculated intraperitoneally at each of the first three transfers of the strain, the majority died in about 2 days with diffuse liver necrosis, which is the usual period of ordinary Rift Valley fever. From the 4th until the 12th passage a certain amount of irregularity regarding the date of death was noticed, and sometimes a number of the mice survived. These latter were subsequently found to have been rendered immune to the ordinary viscerotropic strain of the Rift Valley virus. The mice that did not survive almost all died later than the corresponding animals which had been inoculated intracerebrally. Sometimes the period between inoculation and death extended to 8 or 12 days, and when this occurred nervous symptoms frequently preceded death. These symptoms, similar in nature but more severe than those already noted in the mice which had been inoculated intracerebrally, suggested localization of the virus in the brain. This suggestion was later confirmed by inoculation experiments and by histological examination.

At the 13th passage, however, the activity of the intracerebral passage virus, when inoculated intraperitoneally, underwent a somewhat rapid change, and only one mouse out of a batch of six died, while the five survivors were found to have been immunized. From the 14th passage onwards less than half of the mice inoculated by the intraperitoneal route succumbed, and almost always with cerebral symptoms. Table I gives the details of some of these early passages.

TABLE I.—*Interval in Days Between Date of Inoculation and Death.*

Mice.	2nd passage.		4th passage.		7th passage.		12th passage.		13th passage.		16th passage.	
	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.
1	4	2	2	3	3	6	3	4	3	10 C.	3	8 C.
2	4	2	3	4	3	8	3	8 C.	3	Imm.	3	9 C.
3	4	2	3	4	3	8	3	8 C.	3	„	3	Imm.
4	4	2	3	9	4	Imm.	3	10 C.	4	„	3	„
5	4	2	3	9	4	„	3	12 C.	4	„	3	„
6	4	2	4	9	4	„	3	12 C.	4	„	3	„

i.c. = Intracerebral inoculation; i.p. = intraperitoneal inoculation. Figures indicate interval in days between inoculation and death. Imm. = Immunized; C. = cerebral symptoms noted.

It has thus been possible to create two independent but similar variant strains of the Rift Valley fever virus. In the original strain the most rapid change-over to neurotropism appeared to take place about the 10th intracerebral passage, while in the second it was noted at the 13th passage. Although we have referred to these strains as fixed neurotropic variants, there is still some slight evidence of viscerotropic activity, as shown occasionally by small areas of focal necrosis in the livers of inoculated mice. This applies even to the original strain, which has been passaged some 40 times in partially immunized animals.

It would appear that the neurotropic form of the Rift Valley virus at present lacks some of the stability of the corresponding variant in yellow fever, because when it was passaged six consecutive times in the brains of non-immunized mice a slight but gradual increase in the amount of focal necrosis was seen. Experiments are now being carried out to investigate this point further. Even the neurotropic yellow fever virus may at times produce symptoms suggestive of some degree of liver damage, and can by certain intensive methods, be reconverted back into its viscerotropic form (Findlay and Clarke, 1935*b*).

FACTORS INFLUENCING THE CEREBRAL LOCALIZATION OF THE NEUROTROPIC RIFT VALLEY FEVER VIRUS.

The fact that localization of the virus in the brain only occurred in a proportion of the mice inoculated by the intraperitoneal route, suggested that observations should be made regarding some of the factors affecting this process, and it was decided that these should be considered under the following headings: (1) Effect of injury; (2) effect of age.

Effect of Injury.

It has long been known that certain viruses tend to become localized in an area which has been subject to injury. This fact has been made use of by Sawyer and Lloyd (1931), who have shown that the neurotropic form of yellow fever virus can be induced to localize with great regularity in the brains of mice following intraperitoneal injection of the virus, provided the brain is injured at the same time. For this purpose the injection of a small quantity of starch is generally used. It was decided to study the effect of this method of localization on the neurotropic Rift Valley variant.

Accordingly, a number of mice were anæsthetized and inoculated intracerebrally with 0·03 c.c. of 2 p.c. solution of starch. About half an hour later, these, along with suitable controls, were each injected intraperitoneally with 0·25 c.c. of neurotropic brain emulsion, made by grinding mouse brains in the proportion of one brain to 5 c.c. of saline solution and then lightly centrifuging. This experiment has been repeated several times, and the results obtained have always been similar. In every case the mice which had been treated with starch died with cerebral localization of the virus, while only a proportion of the controls succumbed. Thus, in Rift Valley fever as in yellow fever, localization can be brought about by injury.

Effect of Age.

In general it may be said that cerebral localization of a virus occurs more readily in young individuals, as was shown by Theiler (1930) in the case of yellow fever. In order to find out if age also had an effect in determining the localization of the Rift Valley virus, a number of mice about 6 weeks old were selected, along with a similar number of older mice. These two batches were given an intraperitoneal injection of 0·25 c.c. of the neurotropic brain emulsion, and were kept under observation for 3 weeks; during this period cerebral localization occurred in all the young mice, but only in about half of the older ones. It therefore appears that young mice are more susceptible than older ones to spontaneous cerebral localization following intraperitoneal injection of this virus.

DISTRIBUTION AND FATE OF THE NEUROTROPIC RIFT VALLEY VIRUS FOLLOWING INTRAPERITONEAL INJECTION IN MICE.

The fact that spontaneous localization in the brain occurred in a proportion of the mice inoculated with the neurotropic virus by the intraperitoneal route suggested that observations should be made on the distribution of the virus in the tissues at varying intervals after inoculation. In order to do this a batch of some 30 mice of about the same age were inoculated intraperitoneally with 0·25 c.c. of neurotropic brain emulsion, and then at intervals batches of three were killed and certain of their organs and tissues removed and pooled.

In one such experiment the mice were killed at intervals of 2 hours and 2, 4, 6 and 8 days, and in each case suspensions of the pooled bloods, spleens, livers and brains were inoculated intracerebrally into six mice.

TABLE II.—*Distribution of Neurotropic Rift Valley Virus at Varying Intervals following Intraperitoneal Inoculation.*

	Blood.	Spleen.	Liver.	Brain.
2 hours	6	6	6	0
2 days	6	6	6	0
4 ,,	4	6	6	1
6 ,,	0	5	0	0
8 ,,	0	6	0	3

Figures indicate number of mice out of six dying after intracerebral inoculation of the pooled tissues of three mice killed at the periods indicated.

In the first 2 days the virus seemed to be more or less evenly distributed between the blood, spleen and liver, and absent from the brain, as is shown in Table II. By the 4th day a diminution of the amount of virus present in the blood-stream was noted, although the spleen and liver remained rich in virus. On the 6th day the blood and liver were completely free from virus, which, however, still persisted in the spleen. The 8th day showed that the blood and liver were still free from virus, although the amount in the spleen appeared to be undiminished. On this day, however, a considerable amount of virus was present in the pooled brains, although these mice had not yet developed nervous symptoms.

The fact that the infection had succeeded in reaching the brain in spite of the clearing of the blood-stream two days previously suggested that the virus might not have come there directly from the blood, but that possibly an alternative route may have been employed, such as excretion on to the nasal mucosa and thence by the olfactory nerves to the brain, as has been described by Hurst (1936) in equine encephalomyelitis.

Intranasal instillation.—In a large number of other viruses with neurotropic affinities it has been found that nasal instillation is followed by the development of encephalitic symptoms. The following experiment shows that nasal instillation of the neurotropic strain of Rift Valley fever also leads to encephalitis.

Twenty mice were lightly anæsthetized with ether, and into the nostrils of each mouse were instilled 0·03 c.c. of a 20 p.c. suspension of mouse brain infected with neurotropic Rift Valley fever. The fate of the mice is seen in Table III.

TABLE III.

Number of days after inoculation.	Number of mice dying with nervous symptoms.
2	1
3	0
4	0
5	1
6	4
7	10
8	4

Encephalitic changes were present in the brains of these mice, but histological lesions in the liver were minimal. At death virus was absent from the blood-stream, but constantly present in the brain, while as a rule the spleen also contained small amounts of virus. When monkeys (Findlay, 1931–32) or mice (Francis and Magill, 1935) are inoculated intranasally with the ordinary strain of Rift Valley virus the results are similar to those following subcutaneous inoculation in that mice die with liver necrosis, while monkeys exhibit a short febrile reaction associated with the circulation of virus in the peripheral blood-stream.

The virus of the neurotropic Rift Valley fever, after intranasal instillation, reaches both the brain and certain of the abdominal viscera, as does the neurotropic strain of yellow fever (Findlay and Clarke, 1935*a*). Experiments are at present being undertaken to determine whether the nasal mucosa forms

the only path by which virus can pass from the blood-stream to the brain. It will be remembered that Francis and Magill (1935) found that in man infected with ordinary Rift Valley fever, virus may pass from the blood-stream on to the nasal mucosa.

THE SUSCEPTIBILITY OF OTHER RODENTS.

The viscerotropic strain of Rift Valley fever is pathogenic for a number of other rodents, in addition to mice (Findlay, 1931–32 ; Daubney and Hudson, 1933–34), in all of which it produces liver necrosis. Rabbits and guinea-pigs are practically insusceptible. The pathogenicity of the neurotropic strain was tested in rabbits, guinea-pigs, rats and field voles (*Microtus agrestis*). Rabbits and guinea-pigs proved insusceptible, and the virus did not appear to multiply in their brains ; rats and field voles, on the other hand, died with encephalitic symptoms after intracerebral inoculation, rats in from 5 to 6 days, field voles in 4 to 5 days. At death, in both species, virus was present in the brain but not in the blood-stream.

PATHOLOGY.

The liver.—Although when injected intraperitoneally the protective action of the immune serum on the liver and other viscera was sufficient to prevent death from ordinary Rift Valley fever, it was found in the earlier passages that small aggregates of liver-cells had undergone necrosis, and a picture was presented very similar to that seen in the monkey, sheep and goat, infected with the viscerotropic virus. These small and scattered areas of focal necrosis were infiltrated with lymphocytes and polymorphonuclear leucocytes. After more frequent passage, even in mice given immune serum, occasional cells were found to have undergone necrosis, while here and there the Kupffer cells were swollen, and occasional polymorphonuclear leucocytes were seen in the sinuses (Fig. 1).

The condition was thus very similar to that found in the monkey by Findlay and Stern (1935) and Penna (1936) when yellow fever virus had been inoculated intracerebrally and immune serum intraperitoneally. In rats and field voles infected with neurotropic Rift Valley fever, no microscopical evidence of necrosis could be found.

EXPLANATION OF PLATES.

FIG. 1.—Liver from mouse injected intraperitoneally with immune serum and inoculated intracerebrally with neurotropic Rift Valley virus, showing small area of focal necrosis. Hæmatoxylin and eosin. × 180.

FIG. 2.—Mouse brain showing engorgement of vessels following intracerebral inoculation of neurotropic Rift Valley fever virus. Hæmatoxylin and eosin. × 175.

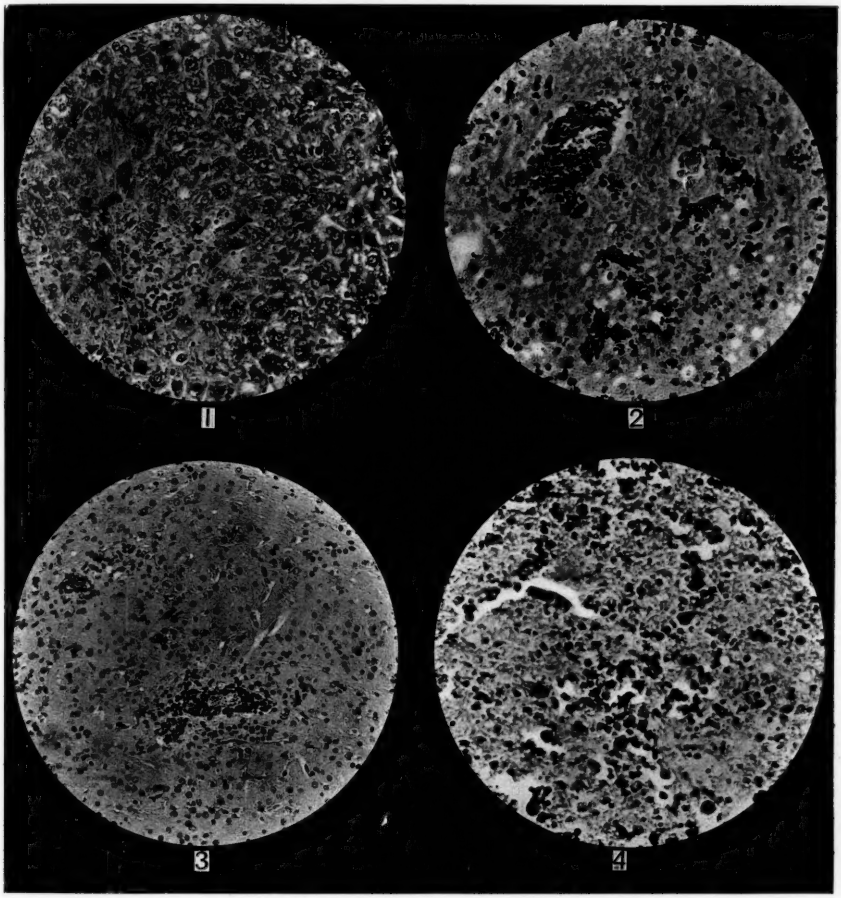
FIG. 3.—Perivascular infiltration around a cerebral vessel in brain of mouse. Hæmatoxylin and eosin. × 150.

FIG. 4.—Small " abscess " in mid-brain. Hæmatoxylin and eosin. × 500.

FIG. 5.—Mouse brain. Inflammatory reaction in ventricle. Hæmatoxylin and eosin. × 145.

FIG. 6.—Degenerated nerve-cells in mid-brain. Hæmatoxylin and eosin. × 600.

FIGS. 7 and 8.—Acidophilic intranuclear inclusions in nerve-cells in mid-brain. Hæmatoxylin and eosin. × 1500.



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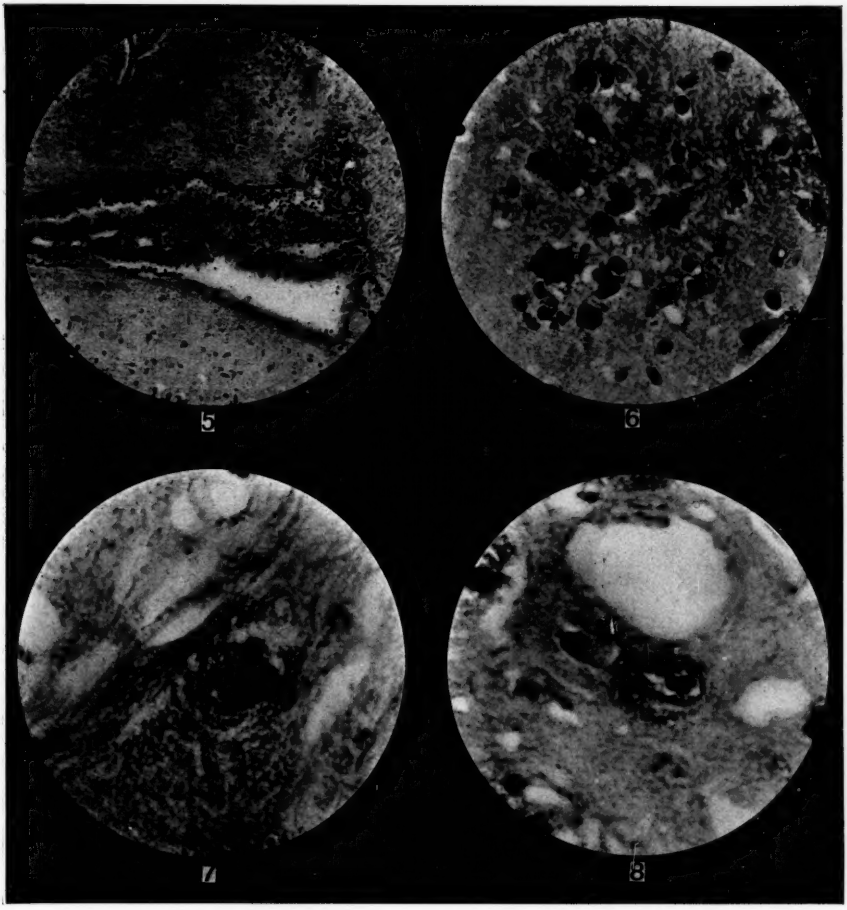

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The other viscera of mice, rats and field voles presented no specific pathological changes.

The central nervous system.—The brains of mice injected intracerebrally with the ordinary viscerotropic virus showed no lesions either macroscopically or microscopically. Both neurotropic variants, on the other hand, produced an intense encephalitis with histological features which recalled those resulting from intracerebral injection of neurotropic yellow fever virus. There were, however, certain differences in the histology of the encephalitis caused by the neurotropic Rift Valley fever virus.

In general, all the mice which died as a result of intracerebral inoculation of either the first or second neurotropic variants of Rift Valley fever virus showed a fairly severe meningeal reaction over the base and vertex of the brain. The whole brain appeared congested, and on microscopical section the vessels in all parts of the brain were found to be engorged with blood (Fig. 2). In contrast to the encephalitis produced by neurotropic yellow fever virus changes in the cerebral cortex were slight; an occasional vessel was seen to be ringed by a single layer of lymphocytes but otherwise the cortex appeared unaffected, there being no evidence of the microglial reaction which was a feature of the yellow fever encephalitis (Fig. 3). The neurotropic virus of Rift Valley fever exerted its toxic effects particularly on the midbrain. Here there was intense perivascular infiltration, and in many sections of this region small masses of lymphocytes not centred on a blood-vessel and giving somewhat the appearance of a tiny abscess were present (Fig. 4). In addition there were a few areas of focal necrosis, though these were not as common in mice as in other animals inoculated with this virus. The small lymphocyte was the predominating type of cell in all the infiltrations, though occasionally polymorphonuclear and plasma-cells were to be recognized. In several mice cellular infiltration was not confined to the vicinity of blood-vessels in the midbrain, and in some cases was found in the ventricle (Fig. 5); it also extended diffusely into the nervous tissue. Many large nerve-cells in the midbrain, and to a less extent in the medulla, were undergoing degeneration. Neuronophagia was often observed, some cells being almost completely surrounded by phagocytic cells which were in the process of devouring them (Fig. 6). The predilection of this virus for the midbrain was striking, as in the medulla, cerebellum and spinal cord lesions were very slight. Occasionally in the mouse there was a true myelitis. In one such animal the tissues of the whole cord were heavily infiltrated with small round-cells; there was intense perivascular infiltration of engorged vessels, and the anterior horn-cells were in varying stages of degeneration.

Small intranuclear inclusions, similar to those observed in the nerve-cells of mice dying from yellow fever encephalitis, were seen in the nerve-cells of the midbrain in many of the mice injected with neurotropic Rift Valley fever virus (Figs. 7 and 8).

Similar, though less severe, histological lesions were found in those mice which had been inoculated intraperitoneally with neurotropic Rift Valley fever virus after intracerebral inoculation of starch; after intraperitoneal injection with the virus without previous damage to the brain only inconsiderable changes were noted histologically.

In rats and field voles dying from Rift Valley fever encephalitis the pathological changes were similar in distribution to those found in mice, the predilection for the midbrain being especially marked. Perivascular infiltration, however, was slight, but many of the nerve-cells were undergoing degeneration, and here and there scattered throughout the brain substance and not centred on a blood-vessel were tiny collections of round-cells, similar to those observed in mice. In the brains of rats and field voles the changes, apart from degeneration of nerve-cells, were often, however, comparatively slight.

DISCUSSION.

By the method of local immunization here described it has been possible to demonstrate in the viruses both of yellow fever and Rift Valley fever a pathogenic potentiality that is normally hidden, and thus to show that both agents belong to the group of pantropic viruses, since they can produce lesions in tissues derived from all three embryonic layers.

A further demonstration is thus given of the resemblance of Rift Valley fever to yellow fever. The close similarity of the clinical symptoms and pathological changes produced by the pantropic strains of the two viruses has already been emphasized. The neurotropic strains also produce the same type of lesions in the central nervous system of the mouse, the primary attack being on the neurones, which in both cases exhibit acidophilic intranuclear inclusions of very similar appearances. In adult mice, inoculated intraperitoneally, the virus does not, as a rule, reach the central nervous system, though the tendency to cerebral involvement is greater in Rift Valley fever than in yellow fever. In very young mice intraperitoneal inoculation invariably leads to death from encephalitis. In addition to the similarities exhibited by the behaviour of the two viruses in the mouse, there is also a similarity in the mode of transmission ; for, although the insect vector of Rift Valley fever is still somewhat uncertain, there is considerable evidence to suggest that aëdine mosquitoes are responsible. In addition to yellow fever, Rift Valley fever exhibits certain analogies with dengue and equine encephalomyelitis. Although the viruses causing these diseases appear to be immunologically distinct, there seems little doubt that they should be classified in a single group.

SUMMARY.

1. By the intraperitoneal injection of immune serum and the intracerebral injection of Rift Valley fever virus into mice it is possible to bring to light the latent neurotropic potentialities of the virus.

2. By continued intracerebral passage in mice a " fixed " strain of neurotropic Rift Valley fever virus can be produced.

3. This virus, when injected intraperitoneally into adult mice, does not give rise to diffuse necrosis of the liver, but immunizes against the pantropic virus.

4. When injected intraperitoneally into very young mice the virus regularly localizes in the central nervous system with the production of encephalitis. In adult mice intraperitoneal inoculation may also in some cases be followed by localization of the virus in the brain.

5. The lesions produced in the brain are those of a true encephalitis associated with degeneration of nerve-cells in which are sometimes seen acidophilic intranuclear inclusions.

6. Rats and field voles (*Microtus agrestis*) succumb to encephalitis, following intracerebral inoculation of neurotropic Rift Valley fever virus. Rabbits and guinea-pigs are apparently insusceptible.

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ON THE IMMUNITY REACTIONS OF IODOPROTEINS.

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Received for publication July 3rd, 1936.

IF horse-serum which has been treated with alkali, nitric acid, nitrous acid or iodine is injected into a rabbit, antibodies, *i. e.* precipitins against alkali-proteins, nitroprotein, azoprotein or iodinated protein are formed (Obermayer and Pick, 1903). Antibodies directed against ordinary unaltered horse-serum do not, however, develop after these injections. Immune reactions to iodinated protein have been studied by Wormall (1930). It is well known that the iodine which is bound by the protein complex reacts especially with the tyrosine groups. This has been proved by Oswald, who succeeded in isolating 3 : 5-diiodotyrosine from both natural and artificial iodoprotein compounds. With the aid of precipitation reactions Wormall was able to confirm the important rôle played by this diiodotyrosine in the combination of iodine and protein. He made use of the so-called inhibition reaction of Landsteiner and v. d. Scheer (1928); here an artificial prozone is caused by addition of the specific

group of the antigen to a precipitation reaction. Wormall found that precipitation obtained with anti-iodoserum and diluted iodinated serum (1 : 100) could be inhibited by the addition of diiodotyrosine. He concluded that therefore the iodinated serum must contain the diiodotyrosine complex.

We have repeated Wormall's experiments and obtained the same results.* Addition of 0·5 millimol p.c. of diiodotyrosine completely inhibited the precipitation of iodoproteins by specific antiserum. Wormall himself has observed that this inhibitory effect seems to be specific, because several other iodine compounds did not have any inhibitory influence upon the precipitation of iodoproteins.

We first examined the question if thyroxine and the closely allied diiodothyronine could influence the precipitation of iodoproteins by specific antiserum.

In carrying out these tests several precautions must be taken. Thyroxine is practically insoluble in water; it is, however, soluble in weakly alkaline liquids. It thus becomes necessary to employ thyroxine in alkaline solution when studying its inhibitive effect on a precipitation reaction. On the other hand, one must be cautious in using an alkaline medium for precipitation reactions because of the strong tendency of the alkali to inhibit the reaction. A solution consisting of 30 c.c. $N/50$ NaOH, 56 c.c. 0·9 p.c. NaCl, aq. destill. ad 100 c.c. hardly retarded precipitation, but would only dissolve 0·2 millimol p.c., *i. e.* 0·155 p.c. thyroxine.

The inhibition of the iodoprotein precipitation by diiodotyrosine does not require a solution as strong as 0·5 millimol p.c., but, as shown by the experiments recorded in Table I, can be obtained with solutions of 0·05 millimol p.c. in an alkaline medium (Table I).

TABLE I.

Immune serum.	Dilution.	Antigen : Iodoprotein cow. Diluted with— Alkaline solution.	Diiodotyrosine in alkaline solution. 0·05 millimol p.c.
Anti-iodoprotein, human	1 : 100	+++	—
	1 : 1000	+++	±
	1 : 10000	++	—

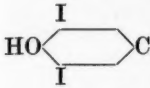
Alkaline solution : 56 c.c. 0·9 p.c. NaCl 30 c.c. $N/50$ NaOH ; aq. ad. 100 c.c.

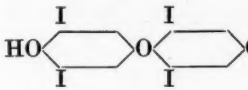
For experiments with thyroxine and diiodothyronine a careful technique is necessary. The pipettes must be warmed beforehand to 40° C. in the incubator. The racks with serological tubes and the tubes containing the solutions must be put in the thermostat before the experiment is started. The entire precipitation reaction must be performed in the thermostat. If these precautions are carefully observed, inhibition experiments with thyroxine and diiodothyronine proceed without difficulty.

The effect of thyroxine and diiodothyronine is shown in Table II. It will be seen that 0·2 millimol p.c. of thyroxine in alkaline solution definitely inhibits the precipitation of iodoprotein by specific antiserum, whereas 0·2 millimol p.c. of 3 : 5-diiodothyronine did not have any influence (Table II).

* For the preparation of the iodoproteins Wormall's modification of the method of Blum and Straus was used. The anti-sera were prepared by four weekly injections in rabbits of 5 c.c. of diiodoprotein. The first two injections were given intravenously, the two following intraperitoneally.

The formulæ for the three substances so far discussed are given below :


3 : 5-diiodotyrosine.


thyroxine.

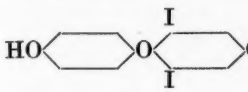
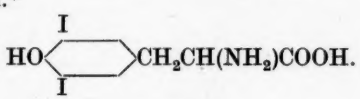
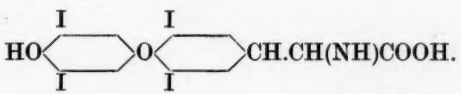

3 : 5-diiodothyronine.

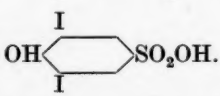
TABLE II.

Immune serum.	Dilution.	Antigen : Iodoprotein human. Diluted with— NaCl 0·9 p.c.	3 : 5-Diiodotyrosine. 0·2 millimol p.c.	Thyroxine. 0·2 millimol p.c.	Diiodothyronine. 0·2 millimol p.c.
Anti-iodoprotein cow	1 : 100	+++	—	—	+++
	1 : 1000	++	—	±	++
	1 : 10000	±	—	—	±

Prof. Landsteiner, when informed by letter about these results, suggested that the specific group which inhibits the precipitation of iodoprotein by specific antiserum would probably be the 3 : 5-diiodo-4-oxy group . This group exists both in diiodotyrosine and in thyroxine, but not in diiodothyronine. His suggestion proved to be correct. The following substances, in all of which the 3 : 5-diiodo-4-oxy group is present, can inhibit the precipitation of iodo-protein by specific antiserum.*

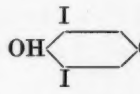
(1) 3 : 5-Diiodotyrosine (Table II) 

(2) Thyroxine (Table II) 

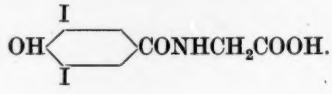
(3) 3 : 5-Diiodo-4-hydroxybenzene sulphonic acid (sozoiodolic acid) (Table III) 

* For these inhibition tests solutions of 0·25–0·5 millimol p.c. were used. 19·5 mg. of 3 : 5-diiodo-4-oxybenzoic acid (molecular weight 390) were neutralized with 2·4 c.c. *N*/50 NaOH. The solution was then made up to 10 c.c. with 0·9 p.c. NaCl, making the concentration 0·5 millimol p.c. In all precipitation tests two drops of the antiserum were added to 0·2 c.c. of the iodoprotein dilution. The test-tubes were placed in the incubator for some hours, after which they were kept at room temperature for about 16 hours. Only the experiments with thyroxine and diiodothyronine solutions must be performed completely in the thermostat at 37° C.

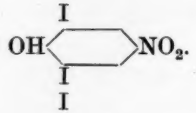
(4) 3 : 5-Diiodo-4-oxybenzoic acid (Table III)



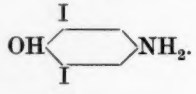
(5) 3 : 5-Diiodo-4-oxyhippuric acid (Table III)



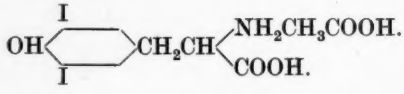
(6) 3 : 5-Diiodo-4-oxy-1-nitrobenzol (Table IV)



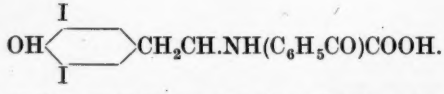
(7) 3 : 5 Diiodo-4-oxy-1-aminobenzene (Table IV)



(8) Diiodotyrosine acetate . .



(9) N-Benzoyldiiodotyrosine .



(1) F. Hoffmann-La Roche & Co., Basel.—(2) *Ibid.*—(3) H. Trommsdorf, Chem. Fabrik, Aachen.—(4) *Organic Syntheses* (1934), **14**, 53.—(5) Prepared by iodinization of p-oxyhippuric acid (*E. Fischer* (1908), *Ber. dtsch. chem. Ges.*, **41**, 2877) with iodo-monochloride (*Vanino* (1921) *Präp. Chemie*, **1**, 64) in glacial acetic acid. The 3 : 5-diiodo-4-oxyhippuric acid showed a M.P. of 221°; N content 3·16 p.c. (theor. 3·16 p.c.); I. content 55·5 p.c. (theor. 56·8 p.c.).—(6) L. Kalb, F. Schweizer, H. Zellner und E. Berthold (1926), *Ber. dtsch. chem. Ges.*, **59**, 1869.—(7) *Idem*, *ibid.*, **59**, 1870.—(8) British Drug Houses, Ltd., London.—(9) Prepared by interaction of benzoylchloride on diiodotyrosine in alkaline solution (Sörensen and Andersen (1908), *Hoppe-Seyl. Z.*, **61**, 290), M.P. of the N-benzoyl diiodotyrosine obtained 179°.

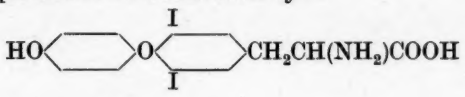
TABLE III.

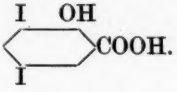
Immune serum.	Dilution.	Antigen : Iodoprotein, human. Diluted with— NaCl 0·9 p.c.	3 : 5-diiodo-4-oxybenzoic acid.	3 : 5-diiodo-4-oxyhippuric acid.	3 : 5-diiodo-4-hydroxybenzene sulphonic acid.	3 : 5-diiodo-2-oxybenzoic acid.
Anti-iodoprotein cow	1 : 100	+++	—	—	—	+++±
	1 : 1000	++±	—	—	—	++±
	1 : 3000	+	—	—	—	+

TABLE IV.

Immune serum.	Dilution.	Antigen : Iodoprotein, human. Diluted with— NaCl 0·9 p.c.	3 : 5-diiodo-4-oxy-1-nitrobenzene.	3 : 5-diiodo-4-oxy-1-aminobenzene.
Anti-iodoprotein cow	1 : 100	++	—	—
	1 : 1000	+++	—	—
	1 : 10000	++	—	—

On the other hand, this precipitation is not inhibited by—

Diiodothyronine (Table II) . 

3 : 5-diiodo-2-oxybenzoic acid (diiodosalicylic acid) (Table III) 

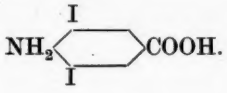
3 : 5-diiodo-4-aminobenzoic acid (Table V) . 

TABLE V.

Immune serum.	Dilution.	Antigen : Iodoprotein, human. Diluted with— NaCl 0·9 p.c.	Diiodotyrosine acetate.	N-benzoyl diiodo-tyrosine.	3 : 5-diiodo-4-amino-benzoic acid.
Anti-iodoprotein sheep	1 : 10	—	—	—	—
	1 : 100	+++	—	—	++±
	1 : 1000	+++	—	—	++±

We next proceeded to ascertain whether or not substitution in the OH-group of the above substances would change the immuno-chemical properties of these compounds.

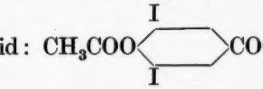
In the first place it could be proved that the acetic acid ester of 3 : 5-diiodo-4-oxybenzoic acid:  is capable of inhibiting the iodoprotein precipitation (Tables VI and VII). The inhibition produced by this ester is, however, somewhat weaker than the inhibition by equimolecular solutions of diiodotyrosine or 3 : 5-diiodo-4-oxybenzoic acid; this can be proved by using solutions of 0·25 millimol p.c. for the inhibition tests (Table VI).

TABLE VI.

Immune serum.	Dilution.	Antigen : Iodoprotein, hog. Diluted with— NaCl 0·9 p.c.	Acetic acid ester of 3 : 5-diiodo-4-oxybenzoic acid. 0·25 millimol p.c.	3 : 5-diiodo-4-oxybenzoic acid. 0·25 millimol p.c.
Anti-iodoprotein (human)	1 : 10	—	—	—
	1 : 100	++++	+±	—
	1 : 1000	+++	—	—

This leads to the question why diiodothyronine does not inhibit the precipitation of iodoprotein by specific antiserum. The formula of diiodothyronine shows that it can be considered as the oxyphenyl ether of diiodotyrosine.

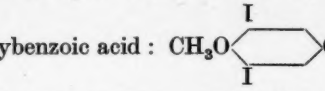
Evidently this substitution in the OH-group of the diiodotyrosine by which no ester but an ether, *i. e.* diiodothyronine, is formed, has completely changed the immunochemical properties of the diiodotyrosine. In view of this fact we synthesized an aliphatic ether of 3 : 5-diiodo-4-oxybenzoic acid. 0·5 millimol p.c. of this substance, *i. e.* the methyl ether of the 3 : 5-diiodo-4-oxybenzoic acid :  , can no longer inhibit the precipitation of iodoprotein by specific antiserum.

TABLE VII.

Immune serum.	Dilution.	Antigen : Iodoprotein, human. Diluted with— NaCl 0·9 p.c.	Acetic acid ester of 3:5-diiodo-4-oxybenzoic acid. 0·5 millimol p.c.	Methyl ether of 3:5-diiodo-4-oxybenzoic acid. 0·5 millimol p.c.
Anti-iodoprotein (cow)	1 : 100	+++	−	+++±
	1 : 1000	++±	−	++±
	1 : 10000	+	−	+

Therefore esterification in the OH-group of the 3 : 5-diiodo-4-oxy group evidently did not change or hardly changed its immunological properties, whereas by substitution in the OH-group with formation of an ether, a substance with fundamentally different immunochemical properties was formed. This explains the fact why diiodothyronine, an aromatic ether of diiodotyrosine, does not possess the immunochemical properties of diiodotyrosine.

It must be noted that most substances containing the 3 : 5 diiodo-4-oxy group give the colour reaction originally described by Kendall and Osterberg as characteristic for thyroxine.

1 c.c. of the solution of one of these substances is mixed with 1 c.c. of 96 p.c. alcohol; 5 drops of concentrated HCl and 5 drops of 1 p.c. sodium nitrite are added. The mixture is boiled and then cooled; after this ammonia is added until it is alkaline. The reaction is positive when a red colour develops.

Later Harington and Barger (1927) demonstrated that this reaction is not only positive with thyroxine, but also with diiodotyrosine. According to our observations iodoprotein also gives a positive Kendall reaction. Furthermore Harington and Barger observed that not only diiodotyrosine but all the substances containing the 3 : 5-diiodo-4-oxy group give this reaction. We can confirm this for *nearly* all the 3 : 5-diiodo-4-oxy compounds mentioned above. It has to be pointed out, however, that the intensity of this Kendall reaction is not the same for all these different substances. In equimolecular concentration diiodotyrosine gives the strongest colour, sozoiodolic acid gives a weaker reaction, whereas with 3 : 5-diiodo-4-oxybenzoic acid and 3 : 5-diiodo-4-oxyhippuric acid the colour is only pink. With 3 : 5 diiodo-4-oxy-1-nitrobenzene the Kendall reaction is even negative; here a strong yellow colour develops, evidently caused by the chromophoric NO_2 group. The Kendall

reaction of 3 : 5-diiodo-4-oxy-1-aminobenzene, which develops with a brown colour, is positive but very weak.

On the other hand, on substitution in the OH of the 3 : 5-diiodo-4-oxy group the Kendall reaction becomes completely negative. Therefore neither the acetyl ester, nor the methyl ether of 3 : 5-diiodo-4-hydroxy benzoic acid, nor diiodothyronine give a positive reaction.* The immunochemical character of the substance may or may not be modified by a substitution in the OH group.

Both ester and ether formed from the group show a negative Kendall reaction, but the acetyl *ester* of 3 : 5-diiodo-4-oxybenzoic acid is capable of inhibiting the precipitation of iodoprotein by specific serum,† whereas the methyl ether of this substance is not.

In close analogy another ether of diiodotyrosine, *i. e.* diiodothyronine (oxyphenyl ether of diiodotyrosine) does not inhibit the iodoprotein precipitation, nor does it give the Kendall reaction.

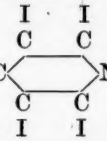
A compound closely allied with the substances mentioned before, *i. e.* 3 : 5-diiodo-4-pyridon‡ :  , also inhibits the precipitation of iodoprotein by specific antiserum (Table VIII) ; but as no 3 : 5-diiodo-4-oxy group is present it does not give a positive Kendall reaction.

TABLE VIII.

Immune serum.	Dilution.	Antigen : Iodoprotein, human. Diluted with— NaCl 0·9 p.c.	Perabrodil.§	3 : 5-diiodo-4-pyridon.	Iothion.
Anti-iodoprotein cow	1 : 100	+++	−	−	+++
	1 : 1000	++±	±	−	++±
	1 : 2000	+±	−	−	+

The inhibition of 3 : 5-diiodo-4-pyridon is somewhat weaker than diiodotyrosine, but of the same order as that produced by the acetic ester of 3 : 5-diiodo-4-oxybenzoic acid.

Perabrodil, 3 : 5-diiodo-4-pyridon-N-acetic acid diethanolamine, a substance derived directly from 3 : 5-diiodo-4-pyridon, also inhibits the iodoprotein precipitation by specific antiserum, but naturally does not show the Kendall reaction.

* It must also be mentioned that diiodosalicylic acid does not give the Kendall reaction.

† The solutions of this acetyl ester used for these inhibition experiments are prepared with the alkaline solution mentioned above. The possibility of hydrolysis of the ester has to be considered, but these solutions usually showed a negative Kendall reaction.

‡ Gift of the I. G. Farben.

§ Mol. weight of perabrodil is 492. The commercial perabrodil consists of a 35 p.c. solution. This was diluted 150 times in order to obtain a solution of 0·5 millimol p.c.

We have also examined an aliphatic diiodo compound, iothion ($CH_2ICHOHCH_2I$). This substance, however, shows no inhibiting influence on the precipitation of iodoprotein by specific antiserum.

SUMMARY.

Precipitation of iodoprotein by specific antiserum is not only inhibited by diiodotyrosine (as proved by Wormall). All substances containing the 3 : 5-diiodo-4-oxy group are capable of this inhibition. Nearly all these substances show a positive Kendall reaction.

Esterification of the OH group of the 3 : 5-diiodo-4-oxy group hardly diminishes this immunochemical quality of the 3 : 5-diiodo-4-oxybenzoic acid. The acetyl ester of this substance still inhibits the precipitation of iodoprotein by specific serum.

On the other hand, after substitution of the OH of the 3 : 5-diiodo-4-oxy group with formation of an ether (methyl ether of 3 : 5-diiodo-4-oxybenzoic acid and 3 : 5-diiodothyronine) no inhibition of the precipitation of iodoprotein can be obtained.

Therefore no parallelism between Kendall reaction and immunochemical properties can be found, as after all substitutions in the OH group of the 3 : 5-diiodo-4-oxy group the Kendall reaction becomes negative.

Another substance without the 3 : 5-diiodo-4-oxy group, *i. e.* 3 : 5-diiodo-4-pyridon, also a substance with a negative Kendall reaction, is nevertheless capable of inhibiting the iodoprotein precipitation.

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THE ALTERATION IN THE BACTERICIDAL POWER OF THE BLOOD IN THE PRESENCE OF INFLAMMATION.

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Received for publication July 6th, 1936.

NORMAL bloods maintain well their bactericidal power *in vitro*, the number of organisms killed at varying intervals after drawing the blood remaining the same for many hours (Alston, 1928). Bloods from cases of bacterial disease behave differently, in that there is a steady deterioration of bactericidal power.

The slide-cell method which Wright, Colebrook and Storer (1923) employed has been used throughout in the experiments described below, the test organism being a recently isolated strain of *Staphylococcus aureus*. The blood to be tested was defibrinated and placed in the water-bath at 37° C., or on the bench at room temperature. The advantage of working at body temperature is that the characteristic changes in the blood are hastened. At intervals specimens of the blood were implanted with suitable numbers of the organism, put into slide-cells and incubated. The number of colonies developing was counted the following day, the average of four cells being taken for each reading. The results in a few typical cases are shown in Table I.

TABLE I.—*Deterioration of Bactericidal Power* in vitro *in Active Bacterial Disease.*

Experiment No.	Nature of case.	Temperature.	Colonies developing at various times after drawing blood.						
1*a*	Malignant endocarditis, febrile	99·6	Time in minutes	10′	60′	100′	135′		
			Colonies developing	16	35	49	57		
1*b*	Same case, afebrile	98·0	Time	50′	68′	100′	150′	180′	
			Colonies	11	21	21	18	18	
2	Tuberculosis of lungs	100·0	Time	15′	45′	78′	148′	199′	235′
			Colonies	20	6	9	11	23	28
3*a*	Lung abscess, not draining	101·0	Time	25′	65′	85′			
			Colonies	43	82	97			
3*b*	Lung abscess draining	98·6	Time	40′	95′	105′	145′		
			Colonies	16	18	15	19		
4	Bronchiectasis	97·0	Time	20′	45′	100′	225′	275′	
			Colonies	8	15	21	31	35	
5	Carcinoma of lung	98·0	Time	25′	65′	95′	155′	230′	275′
			Colonies	7	2	4	2	2	3
6	Chronic nephritis	98·4	Time	25′	65′	95′	125′	455′	
			Colonies	4	3	4	4	4	

It will be seen that blood withdrawn from a case of malignant endocarditis when febrile (1*a*) shows a steady loss of bactericidal power *in vitro*, whereas the sample taken when the patient was afebrile and much improved clinically maintains its power at a constant level. Such deterioration is found in many clinical conditions apart from septicæmias, *e. g.* tuberculosis (2), lung abscess (3*a*) and bronchiectasis (4). Blood from the case of lung abscess shows rapid deterioration as long as the pus is locked up; as soon as the cavity has been emptied and is draining the results approach those given by normal blood. The temperature need not necessarily be raised for the blood to deteriorate (4), but it does not do so in patients severely ill or even dying from non-bacterial disease, as is shown in the cases of carcinoma of the lung (5) and of severe nephritis (6).

This alteration in the bactericidal action of the blood can be produced experimentally by injecting normal people with vaccine. It appears to be confined to the period during which there is evidence of reaction.

TABLE II.—*Deterioration of Bactericidal Power* in vitro *during Reaction after Subcutaneous Vaccine.*

Experiment No.	Time of drawing blood.	Local reaction.	Colonies developing at various times after drawing blood.						
7*a*	17 hours after injection	Present	Time in minutes	15′	45′	75′	105′	135′	165′
			Colonies developing	8	20	26	37	49	60
7*b*	40 hours	Passing off	Time	7′	37′	67′	97′	125′	157′
			Colonies	4	4	7	6	7	8
8*a*	12 hours	Present	Time	20′	45′	65′	115′	155′	
			Colonies	20	16	24	30	58	
8*b*	15 „	Absent	Time	10′	48′	100′	125′		
			Colonies	6	3	4	3		
8*c*	20 „	„	Time	15′	40′	65′	80′	90′	
			Colonies	6	4	2	1	1	

In Experiment 7, a medical student, who was being given a course of mixed anti-catarrh vaccine subcutaneously, was bled 17 hours after the first injection, when there was an extensive local reaction without any marked general disturbance. The specimen (7*a*) shows the steady loss of bactericidal activity seen in the ward cases. A second specimen (7*b*), drawn after 40 hours, when the reaction was passing off, shows much less deterioration. With the next dose of the same vaccine the local reaction was accelerated, being fully developed after 12 hours; at this time the blood (8*a*) behaved like the first specimen. After 15 and 20 hours the blood was again normal (8*b* and 8*c*).

The definite relationship between the presence of reaction and the change in the blood is demonstrated even more clearly by intravenous inoculation of vaccine.

In Experiment 9 a case of arthritis was given T.A.B. vaccine to induce "protein shock"; the rigor began an hour later, and lasted half an hour. Three blood samples were obtained—a control before the injection, a second in the middle of the rigor and the third two hours after the rigor. Here the

TABLE III.—*Effect on Bactericidal Power of Artificially Induced Rigor.*

Experiment No.		Implant.	White count.	Colonies developing at various times after drawing blood.						
9*a*	Control (before injection)	310	7000	Time	12′	50′	92′	170′	225′	
				Colonies	4	6	4	4	5	
9*b*	During rigor	310	3000	Time	15′	50′	100′	155′	210′	255′
				Colonies	55	34	45	42	65	74
9*c*	Two hours after rigor	310	7500	Time	10′	56′	130′	170′	265′	
				Colonies	4	3	4	4	4	
10*a*	Control (before injection)	195	6000	Time	30′	75′	125′	195′	285′	
				Colonies	3	7	3	5	4	
10*b*	As rigor began	195	2400	Time	40′	65′	135′	225′	290′	
				Colonies	80	52	49	50	57	
10*c*	As rigor ended	195	2400	Time	45′	115′	205′	275′	315′	
				Colonies	16	19	26 (leaked)		42	
11	As rigor began	—	—	Time	50′	115′	225′			
				Colonies	69	69	67			

first and third specimens are normal (9*a* and 9*c*), but the specimen taken during the rigor (9*b*) shows the characteristic deterioration, and also a low level of bactericidal activity, which might be due to the leucopenia.

In Experiment 9*b* fewer organisms are killed in the first implant than in succeeding implants; this is frequently seen with both normal and abnormal bloods (2, 5, 10*b*). The phenomenon was also noted by Alston (1928), and appears to be due to some damage connected with defibrination. When blood samples are washed the cells take even longer to recover, in some cases up to two hours (12*a*).

To discover more accurately the time limits between which the change occurs and to attempt to assess the importance of the leucopenia, another case was taken (10). A rigor lasting 20 minutes was produced with T.A.B. vaccine intravenously; the blood was drawn before the injection (10*a*) at the beginning of the rigor (10*b*), and as it ended (10*c*). The differences seen between the specimen taken at the beginning of the rigor and that taken at the end cannot be due to any numerical change in the leucocytes, as the number remained constant. The results suggest that the first change is a marked fall of the bactericidal power followed by some recovery; that this recovery is partial is shown by the eventual deterioration of the third sample, unlike the control, which remains constant. Blood from the same case was examined as another rigor began; the results are shown in Experiment 11, and are similar to those obtained with sample 10*b*.

To determine whether the loss of bactericidal power is dependent upon an alteration of the serum or of the corpuscles Experiment 12 was made. The serum was separated from a sample of normal blood after defibrination and the corpuscles were washed in physiological saline; the same was done with a sample of blood from a case of advanced tuberculosis of the lungs. The blood fractions were then recombined as shown in Table IV.

The results indicate that the serum is at fault, acting as if it contained a toxic substance exerting an inhibitory effect on the bactericidal power of the

TABLE IV.

Experiment No.	Recombined blood consisting of—	Implant.	White count.	Colonies developing at various times after drawing blood.						
12*a*	Normal serum and normal corpuscles	120	2300	Time	15′	90′	155′	210′	270′	345′
				Colonies	27	21	8	9	4	5
12*b*	Patient's serum, normal corpuscles	120	2300	Time	20′	95′	155′	210′	270′	345′
				Colonies	61	39	42	43	39	41
12*c*	Normal serum, patient's corpuscles	120	7700	Time	25′	105′	165′	215′	275′	350′
				Colonies	1	2	1	0	1	2
12*d*	Patient's serum, patient's corpuscles	120	7700	Time	35′	110′	165′	215′	275′	350′
				Colonies	5	4	6	6	13	13

normal leucocytes (12*b*) and, to a lesser extent, on the patient's own cells (12*d*), which function perfectly well in normal serum (12*c*).

This toxic substance was titrated by the method of Fleming for testing the action of antiseptics on blood (Fleming, 1924). The serum from a case of malignant endocarditis was added in progressive dilutions in saline to equal volumes of normal blood. The mixtures were implanted with 100 staphylococci and incubated in slide-cells in the usual way. The results are shown in Table V.

TABLE V.—*Titration of Toxic Substance.*

Experiment No.		Colonies developing at various times after drawing blood.					
13*a*	Patient's blood	Time	60′	120′	200′	260′	320′
		Colonies	2	3	4	9	8
13*b*	Patient's serum in normal blood	Concentration of serum	1/2	1/4	1/8	1/16	Control
		Colonies	51	14	7	1	1

Beyond 1/16 all cells contained only an occasional colony.

DISCUSSION.

The hypothesis that a toxic substance is present in the blood of certain disease cases which, when it is first liberated, exerts an inhibitory effect on the bactericidal power both of normal corpuscles and of the patient's own corpuscles would appear to account for these results. The patient's own corpuscles quickly adjust themselves to this unusual condition in the body, so that their bactericidal power returns to little below the normal. That the adjustment is incomplete is shown by their eventual deterioration *in vitro*. Although the substance will retain its activity for days if kept in the ice-chest, it disappears rapidly from the patient's blood when his inflammation subsides, the corpuscles then returning to their normal condition. The nature of the substance is at present unknown; it has been impossible to initiate the gradual loss of power by adding known substances to the drawn blood, *e. g.* bacterial toxin, autolysed bacteria, filtrate of pus, histamine, etc. Where a change followed the addition of any of these things, it was a reduction once for all of the bactericidal power, similar to the effect of the patient's serum on the normal corpuscles in Experiment 12*b*, or of the onset of the rigor in 10*b* and 11. This

suggests that two factors may be responsible for the gradual deterioration of certain bloods, one the toxic action of a substance liberated during the inflammatory reaction, the other an adjustment of the body to that substance. The deterioration *in vitro* is an expression of the lowered resistance of the corpuscles following their contact with the substance, and its continued action on them. The presence of the substance is not indicated by any of the usual routine blood tests. Parallel investigations of the temperature, the white count, the sedimentation rate, blood culture, blood urea, cholesterol, etc., and the hæmolytic complement show no constant relationship with it. It was found that the best indication of its presence in the patient's blood was his general condition; some indication is given by the temperature, the deterioration usually being shown if this were raised.

SUMMARY.

1. The bactericidal power of normal blood does not fall *in vitro* within six hours.

2. The blood of patients with active bacterial disease shows a steady loss of bactericidal power.

3. Normal persons, when given vaccine, show a similar change during the stage of reaction.

4. Blood drawn during a rigor shows one of two changes: if it is drawn at the beginning of the rigor there is a constant low level of bactericidal power without further loss on standing; if it is drawn later it shows a bactericidal power below normal, and a progressive loss *in vitro*.

5. Blood drawn after the rigor is normal.

6. A mixture of serum taken from a patient with active bacterial disease or with an artificially produced reaction and normal blood-cells shows a constant low bactericidal power with no further deterioration.

My thanks are due to my colleagues in the Inoculation Department, St. Mary's Hospital, for their assistance and criticism, especially to Prof. A. Fleming, Dr. John Freeman and Dr. C. B. Dyson. Without the kind co-operation of Prof. Langmead, Dr. Reginald Miller and other clinicians at St. Mary's this investigation would have been impossible.

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SOME PROPERTIES OF THE CAPSULE OF *PASTEURELLA SEPTICA*.

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Received for publication July 17th, 1936.

Past. septica organisms are usually described as non-capsulated, but there have been a few reports to the contrary. Thus, Gózony (1913), by a special technique involving the use of indian ink, demonstrated capsules around *Past. septica* isolated from fowls and swine, and Carpano (1913), staining exudates from infected animals with carbolized crystal violet or carbol-fuchsin, claimed to have found them around similar organisms from swine and horses. In 1914 Rowlands, using a special hanging-drop technique similar to Gózony's, discovered a peculiar envelope around *Past. pestis*. Schütze (1932*a*) confirmed this observation and noted, in addition, that the so-called envelope was formed at 37° C., but not at 20° C., and that organisms with it had a more complex antigenic structure than those without it.

The object of this paper is to describe the conditions governing the appearance of an envelope around *Past. septica* and to point out its significance in relation to antigenic structure. Most of the work has been done with strains isolated from fowls, but strains from cattle, sheep, pigs, rabbits, rats, mice, etc., have been included in numbers sufficient to show that the essential facts are the same for all. These strains all conformed to the typical description of *Past. septica* (Topley and Wilson, 1929), *i. e.* fermenting glucose, mannitol and sucrose and some other sugars with the formation of acid only, and producing indole from peptone water. Atypical strains, such as those described by Jones (1932), have not been examined.

The exact nature of the envelope is, perhaps, still open to conjecture. The early workers, without question, regarded it as a capsule, and it seems probable that this view is correct. It is true that the usual capsule stains are ineffective in demonstrating it, but since capsules in the various bacterial genera are chemically very different, this would hardly seem convincing evidence. Moreover, the demonstration of some antigenic function associated with the presence of envelope suggests a similarity to capsules of other species. In view of this evidence, therefore, it is proposed, at least for the present, to refer to this structure as a capsule.

* The work reported in this paper is a continuation of investigations begun at the Research Institute in Animal Pathology, Royal Veterinary College, London.

TECHNIQUE.

To demonstrate the capsule a technique similar to that used by Schütze has been adopted. The organisms are suspended in a loopful of 0·85 p.c. NaCl or of distilled water on a slide, a loopful of indian ink is added, a coverslip dropped on, and the preparation examined with a 1/12-in. objective, suitably-graded light being used. When a brand of indian ink known as "Simplon Black" was used the best results were obtained with a 1 in 2·5 dilution. Stronger or weaker concentrations of saline, 6·0 p.c. glucose and other solutions, as recommended by Butt, Bonynge and Joyce (1936), have been tried as diluents, but very little difference has been observed. By this method there is seen round each capsulated organism a relatively wide area uninvaded by the ink particles; organisms without capsules show no such uninvaded area. The capsules may also be demonstrated by a modification of the method introduced by Baker (1920). The bacteria are suspended in indian ink as for coverslip preparations; the mixture is then spread out over a wide area of slide and allowed to dry in air; when dry the film is fixed with heat and stained with a suitable stain. Films stained with hot carbol-fuchsin for a few minutes show a reddish-black background with the bacteria stained red, and, with capsulated organisms, an unstained surrounding space. Under no conditions tried so far does the capsules stain with the usual special stains, *e. g.* those of Hiss and Muir.

CONDITIONS GOVERNING THE APPEARANCE OF THE CAPSULE.

Several variants of *Past. septica* have been described (see, for example, de Kruif, 1921, 1922; Webster and Burn, 1926; Hughes, 1930), but for the purposes of this work it is sufficient to note that virulent and avirulent forms exist. Virulent organisms alone show the capsule; under no conditions do avirulent organisms show it. This important observation was, in fact, made some years ago by Manniger (1919), but its significance has been overlooked by all subsequent workers. Further, the degree of virulence is to some extent associated with size of capsule, organisms showing a wide capsule being more virulent than those with a relatively narrow one. Table I shows the results of experiments demonstrating these points. These experiments were conducted as follows:

Pure avirulent "blue" and virulent "fluorescent" variants (see Hughes, 1930) of *Past. septica* from fowls were obtained by a continuous process of colony selection extending over many months; they were maintained in this state by plating and picking colonies at weekly intervals. Cultures of these variants were grown for 24 hours at 37° C. on serum-agar slopes. The growths were then washed off with 0·85 p.c. saline and standardized by opacity to tube No. 1 of Brown's Opacity Standards. From this, serial 1 in 10 dilutions were made in saline, using a separate pipette for each dilution. 0·5 c.c. amounts of the various dilutions were inoculated intraperitoneally into mice and the results recorded after three days.

It may be seen that the capsulated organisms are very much more virulent than uncapsulated ones, and that organisms with a wide capsule are distinctly more virulent than those with a narrow one.

TABLE I.—*Showing the Difference in Virulence between Capsulated and Uncapsulated* Past. septica *Organisms, as Demonstrated by the Numbers of Mice Surviving Intraperitoneal Injection of Various Dilutions.*

Dilutions inoculated (0·5 c.c. intraperitoneally).	Strain 1.				Strain 2.			
	Capsulated (wide).		Uncapsulated.		Capsulated (narrow).		Uncapsulated.	
	Inoculated.	Survived.	Inoculated.	Survived.	Inoculated.	Survived.	Inoculated.	Survived.
Undiluted	2	0	2	2	2	0	2	1
1 : 10^3	2	0	2	2	2	0	2	2
1 : 10^4	2	0	2	2	2	0	2	2
1 : 10^5	2	0	2	2	2	0	2	2
1 : 10^6	2	0	2	2	2	0	2	2
1 : 10^7	2	0	2	2	2	2	..	..
1 : 10^8	8	0	..	..	2	2	..	..
1 : 10^9	8	2	..	..	2	2	..	..
1 : 10^{10}	8	8	..	..	2	2	..	..

The temperature of incubation is as important for *Past. septica* as Schütze found for *Past. pestis*. The optimum is 37° C.; above and below this temperature the capsule tends not to form; at 20° C. or below and at about 40° C. or above the organisms remain uncapsulated. The time of incubation is also important. After 24 hours' growth at 37° C. the capsule is fully developed; after 48 hours it is still present, but is distinctly smaller; after 72 hours it is generally absent, a few strains only retaining it until the fourth day.

The capsule is formed on most media. Virulent organisms have been examined after growth on plain agar, serum-agar, blood-agar, Fildes's agar, Loeffler's serum and in plain broth; in all cases capsule could be demonstrated; moreover, while the amount of growth varies according to the medium capsule size does not.

The capsule is very heat labile. If saline suspensions are placed in boiling water it disappears almost at once; at 56° C. it is demonstrable for 20 to 30 minutes, after which it rapidly diminishes in size until, after 45 to 60 minutes, it has practically disappeared.

SIGNIFICANCE OF CAPSULE IN RELATION TO ANTIGENIC STRUCTURE.

The antigenic structure of *Past. septica* has been found to be similar in essentials to that of *Past. pestis*. Both the virulent and avirulent variants possess a common somatic antigen, the virulent variant possessing an additional antigen associated with the presence of capsule. The evidence supporting this statement is briefly as follows:

(*a*) With suspensions of avirulent organisms heated to 56° C. for 30 minutes and of virulent organisms heated to 100° C. for 60 minutes (*i. e.* with capsule destroyed) and sera prepared with these, it has been shown by means of complete cross absorption tests, *i. e.* the mirror test, that the two types of organism are antigenically alike.

(*b*) A serum prepared against virulent organisms heated to 56° C. for 30 minutes (*i. e.* with capsule present) and absorbed with a dose of avirulent organisms sufficient to remove all the common somatic antibody still agglutinates the virulent organism to titre. Complement-fixation tests give the same result.

An example of these results is shown in Table II.

TABLE II.—*Demonstrating the Antigenic Structure of* Past. septica.

Serum prepared against—	Absorbed with—	Tested against—	Titre.
..	Unabsorbed	..	160
Avirulent organisms	Avirulent organisms	Avirulent organisms	0
..	Virulent organisms heated to 100° C.	..	0
..	Unabsorbed	..	160
Virulent organisms heated to 100° C.	Avirulent organisms	Virulent organisms heated to 100° C.	0
..	Virulent organisms heated to 100° C.	..	0
Virulent organisms heated to 56° C.	Unabsorbed	..	16
..	Avirulent organisms	Virulent organisms heated to 56° C.	16
..	Virulent organisms heated to 56° C.	..	0

Since virulent organisms in suspension incubated at 56° C. for 1 hour or at 37° C. for 24 hours tend to lose their capsules, it was deemed advisable to leave the agglutination tests at room-temperature, readings being taken after 24 hours. Ice-box temperature was tried, but had no advantage.

The titre of the capsule antibody is always very much lower than that of the somatic antibody. Moreover, it is interesting to note that rabbits immunized with virulent organisms heated to 56° C. for 30 minutes develop demonstrable antibodies, causing agglutination of avirulent organisms some time before those causing agglutination of the fully capsulated organism.

Although no attempt has been made so far to classify *Past. septica* on serological grounds, it may be expected that classification will depend largely upon differences in the capsular antigen. It is, however, already evident that there is more than one type of the somatic antigen ; this has been demonstrated in organisms isolated from cattle and fowls.

DISCUSSION.

The extreme lability of the capsule of *Past. septica* probably accounts for the fact that its presence and significance are apparently little known. Both de Kruif and Hughes stated that there was no difference in the antigenic structure of virulent and avirulent organisms, but their methods of preparing suspensions or performing the serological tests perhaps resulted in destruction of the capsule. A perusal of the literature reveals that overheated cultures have frequently been used for the purpose of studying the antigenic structure of

Past. septica. On the other hand, several workers have noted that certain strains of *Past. septica* are inagglutinable with ordinary sera and even with homologous serum. The author has frequently observed this, and, in fact, noted some years ago that if broth cultures of these strains were incubated for 3 days at 37° C. they became agglutinable. The explanation of this is now quite clear. The sera then in use contained only somatic antibody. Strains inagglutinable with such sera are inagglutinable in virtue of capsule formation. After incubation for 3 days the capsule disappears, exposing the somatic antigen, so rendering the bacteria agglutinable.

The practical importance of the observations reported in this paper, apart from affording a basis of classification, will depend on the relation of the capsular antigen to immunity. Experiments designed to test this point are now being made and will be reported in full at a later date. The available evidence suggests that the envelope is of considerable importance, and in view of Schütze's (1932*b*) results with *Past. pestis* this is perhaps not surprising.

It is interesting to note that the capsule of *Past. septica* has properties similar to those of the Vi antigen in the Salmonella group. Thus both for the formation of Vi antigen and capsule the optimum temperature is 37° C., both are very heat-labile, both are weakly antigenic, both account for apparent inagglutinability and both are factors concerned with virulence.

SUMMARY.

1. A capsular envelope similar to that described in *Past. pestis* is shown to be present round virulent but not avirulent organisms of the *Past. septica* species.

2. This capsule appears after incubation for 24 hours at 37° C. Incubation at higher or lower temperatures tends to inhibit its formation. Incubation for more than 24 hours results in loss of capsule substance. The type of medium used has little or no effect.

3. The capsule is very heat-labile, being destroyed at 56° C. in 45 to 60 minutes, and almost instantaneously at 100° C.

4. Antigenically, virulent organisms are more complex than avirulent ones. In both there is a common somatic antigen, but the virulent organism has an additional antigen associated with the capsular envelope.

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THE INFLUENCE OF CERTAIN BACTERIAL FRACTIONS DERIVED FROM *BACT. AERTRYCKE* ON THE OXYGEN UPTAKE OF RABBIT BRAIN AND MUSCLE SUSPENSIONS.

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Received for publication July 22nd, 1936.

STUDIES of the tissue respiration of animals suffering from a bacterial infection or of the modifications in the oxygen uptake which the admixture of bacteria or of bacterial products may produce in tissues isolated from the normal animal, have not been numerous. Frei and Froebel (1935) recorded that the isolated liver and kidney of guinea-pigs infected with Gaertner's bacillus, fowl cholera, malignant œdema or anthrax, showed an increased respiration-rate. Liver and kidney from normal animals, when mixed with certain bacterial filtrates, also showed an increased oxygen uptake. Killian and Schlossman (1928), in manometric experiments on tissue respiration found diphtheria toxin to be without effect on goose erythrocytes, rat brain and guinea-pig liver, and tetanus toxin had no demonstrable influence on erythrocytes or brain-tissue. Keller (1928), in similar experiments with tuberculin and extracts of the tubercle bacillus, could demonstrate no effect on the respiration of kidney, spleen or liver. The experiments now recorded deal with the effect on the oxygen uptake of normal rabbit brain and muscle suspensions of bacterial fractions derived from *Bact. aertrycke*. Some of these bacterial fractions were described in regard to their preparation, chemical properties and immunizing power by Raistrick and Topley (1934). Further chemical and immunological studies of these fractions are shortly to be recorded by these authors and their collaborators. Some of these bacterial fractions, when injected into rabbits, were found by one of us (Delafield, 1934) to produce the blood-sugar changes and toxic effects which the injection of the dead bacterial cells had been shown to produce (Delafield, 1931, 1932). Little is known about the mechanism whereby these changes in the carbohydrate metabolism are brought about. Evans and Zeckwer (1927) found that rabbits whose left suprarenal had been removed and the right denervated did not manifest the usual hyperglycæmic response when killed *Bact. coli* or *Proteus vulgaris* were injected intravenously, and concluded from their experiments that this response was due to central stimulation of the suprarenals by way of the splanchnic nerves. Menten and Manning (1924, 1925) described lesions occurring in rabbits dying of enteritidis-paratyphoid B infections. They found lesions in the kidneys, liver, spleen, suprarenals and pancreas, but Menten (1926) noted also a perivascular round-celled infiltration and marked degeneration of the ganglion cells in the cerebral cortex and cerebellum.

The experiments recorded below were undertaken to study the possible effect of the *Bact. aertrycke* fractions in modifying tissue respiration *in vitro*, in the hope of making some contribution towards the elucidation of the mechanism of toxic bacterial action. Any changes produced by bacterial fractions in the oxygen uptake of isolated tissues, though not necessarily to be regarded as occurring also in the living animal, might yet throw some light on those properties of these bacterial fractions by virtue of which they exercise their toxic action.

EXPERIMENTAL.

The Bacterial Fractions.

The relationship of the fractions used is indicated below.

Toxic :

(1) Dried bodies of *Bact. aertrycke* grown on agar, extracted with acetone and dried with alcohol and ether.

(2) Crude F68, soluble in water, obtained from the bacterial bodies by tryptic digestion followed by repeated fractionations with alcohol at a concentration of 68 p.c.

(3) F68, soluble in water, obtained from crude F68 by precipitating the calcium contained in the latter by the equivalent amount of oxalic acid and further alcohol precipitation.

(4) F68/P, insoluble in water, obtained from F68 as a precipitate, by heating with $N/10$ acetic acid at 100° C.

Non-toxic :

(5) F68/D + L, soluble in water, is left in solution after the acetic acid treatment of F68 and is precipitated from solution with alcohol.

(6) F95, soluble in water, the precipitate obtained from the tryptic digest by increasing the alcohol concentration to 95 p.c., after the removal of the crude F68 fraction.

The Tissue Suspensions.

Only brain and muscle suspensions from rabbits were examined. Brain was removed within a few minutes of the killing of the animal by a blow on the neck, ground up in a mortar without sand to a fine cream and suspended in Ringer solution. This suspension could be readily pipetted and a very good measure of agreement was obtained in the dry weight of tissue present in equal volumes. Muscle from the back of a recently-killed rabbit was finely chopped and washed several times in a large volume of tap-water. After the water had been squeezed out the muscle was ground up in a mortar to a fine paste and again suspended in tap-water. Some of this suspension was then poured on to muslin and the retained muscle mass was rubbed with a watch-glass. The smaller particles passed through the fabric and the tissue could be collected from the under surface in so fine a state of division that it could be pipetted accurately when resuspended in tap-water.

Measurement of Oxygen Uptake.

This was determined in the Barcroft type of micro-respirometer. Six manometers were shaken at a time at a rate of about 120 shakes per minute at a temperature of about 37·5° C. The Ringer solution used contained NaCl 9·0 g., $CaCl_2$ 0·24 g. and KCl 0·42 g. per litre, and, when needed, 0·2 p.c. of glucose was added. Succinate and lactate as substrates were used as neutralized (pH 7·4) solutions in concentrations of $M/6·5$ and $M/5$ respectively. Phosphate buffer at pH 7·4 was always added to each cup. Carbon dioxide was absorbed by 20 p.c. potash.

The effect of a bacterial fraction was determined by comparing the total oxygen uptake of the tissue suspension alone, and when mixed with the fraction under test. Two or three manometers were used for each group, so that the error inherent in comparing single observations might be lessened. In a typical experiment three manometers were thus set up :

Left-hand cups : 2 c.c. Ringer, 0·5 c.c. phosphate buffer, 0·5 c.c. lactate.
Right-hand cups : 1 c.c. brain suspension in Ringer, 1 c.c. Ringer, 0·5 c.c. phosphate buffer and 0·5 c.c. lactate.

The readings were compared with those given by three manometers, which had been prepared in the same way but with the addition of 3·0 mg. of the bacterial fraction to each cup. The manometers were read at regular intervals for periods of one to three hours.

The Toxicity of the Fractions.

Crude F68, F68, F68/P and the bacterial bodies almost invariably produced definite illness and a marked rise in the blood-sugar of rabbits when injected intravenously in a dose of 2·0 mg. or less per kg. body-weight. The rise might be as great as 200 p.c. two hours after injection and was followed by a hypoglycæmia of variable degree when the blood was examined after twenty-four hours. The comparative effect of these fractions has not been determined on rabbits, but there was merely a general impression that F68/P was less toxic than the other fractions. The lethal dose of F68 has not been determined, but 2·0 mg. per kg. body-weight was sometimes fatal. Fractions F95 and F68/D + L produced no blood-sugar changes and were without obvious effect on the animal.

The Effect of Dried Bacterial Bodies on the Oxygen Uptake of Rabbit Brain Suspensions in Glucose.

The bodies, which in 3·0 mg. amounts had no oxygen uptake in the presence of 0·2 p.c. glucose, had no significance effect on brain suspensions in two experiments, the differences being 5 p.c. and 1 p.c. (Table I).

The Effect of F/68 on the Oxygen Uptake of Rabbit Brain Suspensions.

F/68 alone had no demonstrable oxygen uptake in 3·0 mg. amounts in the presence of glucose or succinate.

Without added substrate.—Brain suspensions in plain Ringer had a considerable oxygen uptake which was, however, not greatly altered in any of four experiments by the addition of F68 in a final concentration of 1·0 mg. per c.c. of fluid. The tests with the bacterial fraction always showed a slightly higher uptake amounting in one experiment to 8 p.c. The results of two experiments are given in Table I.

TABLE I.—*The Effect of the Toxic Fractions and Bodies of* Bact. aertrycke *on the Oxygen Uptake of Rabbit Brain Suspensions in the Presence of* 0·2 *p.c. Glucose. Final Concentration of the Fractions,* 1·0 *mg. per c.c.*

		Added substrate.	Oxygen uptake in c.mm. 20 min.	40 min.	60 min.	Percentage effect on oxygen uptake.
1. Brain alone	(3)	None	87	164	232	+ 2·0
„ + F68	(3)	„	91	172	236	
2. „ alone	(3)	„	98	180	258	+ 4·0
„ + F68	(2)	„	107	195	268	
3. „ alone	(2)	Glucose 0·2 p.c.	36	68	102	+ 62
„ F68	(2)	„	61	116	166	
4. „ alone	(2)	„	42	80	118	+ 50
„ + F68	(2)	„	59	120	178	
5. „ alone	(3)	„	85	165	248	+ 47
„ crude F68	(3)	„	116	235	365	
6. „ alone	(3)	„	74	146	211	+ 11
„ + F68/P	(3)	„	80	159	234	
7. „ alone	(3)	„	109	212	311	+ 24
„ + F68/P	(3)	„	135	263	386	
8. „ alone	(3)	„	68	133	199	±1
„ + bacterial bodies	(3)	„	64	129	197	

The figures for oxygen uptake are the averaged results of the readings of the number of manometers (shown in brackets) used.

With glucose.—The effect of F68 on the oxygen uptake of brain suspensions in the presence of 0·2 p.c. glucose was examined many times and in every instance this bacterial fraction caused a decided increase in the oxygen taken up. This increase in different tissue suspensions ranged from 40 to about 90 p.c. and was manifest from the beginning of the experiment. Examples are given in Table I. The crude F68 was tested only once, and at a concentration of 1·0 mg. per c.c. was found to increase the uptake by 47 p.c. The smallest concentration of F68 which would produce a demonstrable effect under these conditions was about 0·1 mg. per c.c. Chart I records one of these experiments in which 1·0 mg. per c.c. increased the respiration by 89 p.c. and 0·1 mg. produced an increase of 24 p.c. but 0·01 mg. had no demonstrable effect.

With lactate.—In the presence of lactate in a final concentration of 0·37 p.c. of sodium lactate F68 produced some increase in the oxygen uptake, but the

effect was not so definite as with glucose. In two experiments in Table II the increase amounted to 13 and 25 p.c.

With succinate.—This substrate was tested to provide data for comparison with the results obtained later with washed muscle and succinate. In the presence of succinate in a final concentration of 0·4 p.c., F68 produced

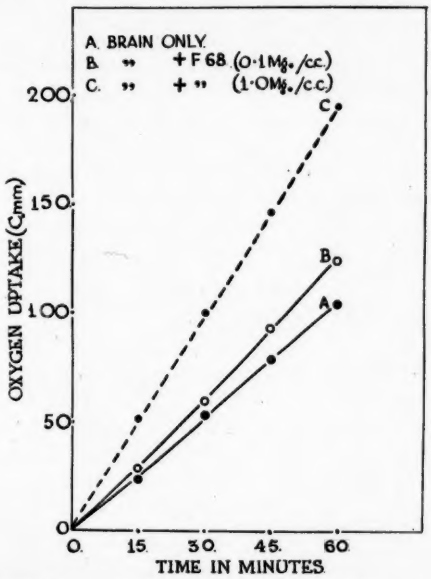

CHART 1.—The effect of F68 in increasing the oxygen uptake of a brain suspension in the presence of 0·2 p.c. glucose. The amount of dry weight of brain was 30·0 mg. in 1 c.c. of brain suspension.

TABLE II.—*The Effect of F68 on the Oxygen Uptake of Rabbit Brain Suspensions in the Presence of Lactate or Succinate. Final Concentration of Bacterial Fraction was 1·0 mg. per. c.c.*

		Added substrate.	Oxygen uptake in c.mm. 20 min.	40 min.	60 min.	Percentage effect on oxygen uptake.
1. Brain alone	(3)	Lactate 0·37 p.c.	66	123	177	+ 13
,, + F68	(3)	,,	72	138	201	
2. ,, alone	(3)	Lactate	56	107	160	+ 25
,, + F68	(3)	,,	69	134	201	
3. ,, alone	(3)	Succinate 0·4 p.c.	124	220	302	− 13
,, F68 +	(3)	,,	112	195	260	
4. ,, alone	(3)	Succinate	161	296	413	− 4
,, + F68	(3)	,,	160	289	397	

The figures for oxygen uptake are the averaged results of the readings of the number of manometers (shown in brackets) used.

no increase of the respiration of the brain suspension. In two experiments there was an inhibition of 4 p.c. in one instance and of 13 p.c. in the other. The later experiments on brain washings suggest that even the smaller of these differences may have been significant. Two results are recorded in Table II.

The Effect of F68/P on the Oxygen Uptake of Rabbit Brain Suspensions with Glucose.

F68/P produces some hyperglycæmia and toxic effects on intravenous injection into rabbits, but it is only very slightly toxic for mice. This fraction, finely ground and suspended in a concentration of 1·0 mg. per c.c., also increased the oxygen uptake of brain in glucose, but not to the same extent as F68. In three experiments two of which are recorded in Table I, the increases amounted to 11, 16 and 24 p.c.

The Effect of the Non-Toxic F68/D + L and F95 on the Oxygen Uptake of Rabbit Brain Suspensions with Glucose.

These fractions were without toxic effect on the animal and produced no hyperglycæmia. F68/D + L had no significant effect in modifying the oxygen uptake of a brain suspension in the presence of glucose when present in a final concentration of 1·0 mg. per c.c. F95 had no definite accelerating effect on the respiration of brain in glucose. In two experiments there were alterations of + 4 p.c. and − 2 p.c., whereas in the third trial there was an increase of about 10 p.c. in the average. Some results are given in Table III.

TABLE III.—*The Effect of the Non-Toxic Fractions on the Oxygen Uptake of Rabbit Brain Suspensions in the Presence of Glucose. Final Concentration of the Bacterial Fractions was 1·0 mg. per c.c.*

	Added substrate.	Oxygen uptake in c.mm. 20 min.	40 min.	60 min.	Percentage effect on oxygen uptake.
1. Brain alone (3)	Glucose 0·2 p.c.	69	134	193	
„ + F68/D+L (3)	„	67	130	189	− 2
2. „ alone (3)	„	79	153	226	
„ + F68/D+L (3)	„	78	148	219	− 3
3. „ alone (2)	„	78	165	238	
„ + F95 (2)	„	85	177	248	+ 4
4. „ alone (3)	„	84	169	251	
„ + F95 (3)	„	82	165	247	− 2

The figures for oxygen uptake are the averaged results of the readings of the number of manometers (shown in brackets) used.

Experiments with Washed Brain and Brain Washings.

The observation that the effect of F68 on brain suspensions varied according to the substrate used, namely, acceleration with glucose and a possible

inhibition with succinate led to these experiments. Cohen and Gerrard (1934) found that the cell-free sol obtained by cytolysing brain-tissue in distilled water, dehydrogenated succinate, but not glucose, and it was hoped that by simple washing of brain in Ringer it might be possible to secure at any rate a partial separation of the enzymes responsible for glucose and for succinate oxidation and so demonstrate more clearly the effect of F68 in the presence of these substrates. Furthermore, this experiment, by washing away some of the soluble matter from brain-tissue, would demonstrate whether or not there was evidence that F68 exercised its stimulating effect in the presence of glucose on the cellular elements of the brain.

Rabbit brain was removed within a few minutes of killing and ground up without sand to a fine cream, shaken up in three to four times its volume of Ringer and spun for fifteen minutes. The opalescent supernatant was decanted and these brain washings were tested the same day for their oxygen uptake. The centrifugate was washed again twice with about three to four times its volume of Ringer, resuspended in Ringer or glucose-Ringer and tested at once.

TABLE IV.—*The Effect of F68 on the Oxygen Uptake of Washed Brain Suspensions. Concentration of the Bacterial Fraction was 1·0 mg. per c.c.*

		Added substrate.	Oxygen uptake in c.mm. 20 min.	40 min.	60 min.	Percentage effect on oxygen uptake.
1. Brain alone	(3)	Glucose 0·2 p.c.	39	70	100	
,, + F68	(3)	,,	39	82	126	+ 26
2. ,, alone	(3)	,,	49	97	147	
,, + F68	(3)	,,	66	123	183	+ 24
3. ,, alone	(3)	Lactate 0·37 p.c.	77	153	226	
,, + F68	(3)	,,	88	176	256	+ 13
4. ,, alone	(3)	,,	69	136	202	
,, + F68	(3)	,,	79	154	227	+ 12
5. ,, alone	(3)	Succinate 0·4 p.c.	72	136	195	
,, + F68	(3)	,,	75	133	183	− 6
6. ,, alone	(3)	,,	76	127	175	
,, + F68	(3)	,,	65	114	156	− 11

The figures for oxygen uptake are the averaged results of the readings of the number of manometers (shown in brackets) used.

Effect of F68 on the oxygen uptake of washed brain.—This bacterial fraction was found in four experiments, two of which are recorded in Table IV, to increase the oxygen uptake of washed brain in glucose in one hour by between 18 and 34 p.c. It was also noted that if the experiment was allowed to proceed longer than one hour the difference tended also to increase. In one experiment the percentage differences at the end of one, two and three hours were 22, 40 and 55 respectively. In two other experiments this difference increased between the end of the first and second hours from 34 to 40 p.c. and from 18 to 27 p.c. The fourth experiment was not continued longer than one hour. With lactate as the substrate the bacterial fraction increased the oxygen uptake by about

12 p.c., and in the presence of succinate there was again evidence of an inhibition amounting in two experiments to 6 and 11 p.c.

Effect of F68 on the oxygen uptake of brain washings.—The brain washings were used in 1·0 c.c. amounts and the dry weight of the solids in this volume, after deducting the weight of the Ringer salts, was about 10·0 mg. in each of the experiments. With added lactate or glucose these washings had no demonstrable oxygen uptake and the presence of F68 did not have any demonstrable effect. A very vigorous oxygen uptake was, however, shown in the

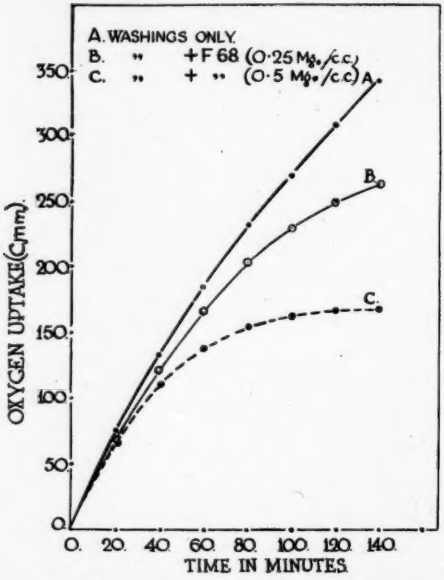

CHART 2.—The effect of F68 in inhibiting the oxygen uptake of brain washings in the presence of 0·4 p.c. succinate. The amount of dry material in 1·0 c.c. of washings was 10·0 mg.

presence of succinate, which was almost completely inhibited by the presence of 1·0 mg. per c.c. of the bacterial fraction. One experiment gave the following :

Oxygen uptake of 1·0 c.c. of brain washings in succinate, 140 c.mm.
" " " " " " + 1·0 mg. per c.c. of the bacterial fraction 15 c.mm.

which was an inhibition of 89 p.c.

Chart 2 gives the results of another experiment with smaller amounts of the fraction. Each curve is the mean of two concordant results. The experiment was continued for over two hours and the curves of the oxygen uptake in the presence of the fraction show a very marked flattening as time goes on. At one hour the percentage inhibitions produced by 0·5 mg. and 0·25 mg. per c.c. are 25 and 9 p.c. respectively, but at two hours these have become 46 and 19 p.c.

The Effect of the Fractions on the Oxygen Uptake of Washed Rabbit Muscle Suspensions in Succinate.

In the presence of lactate or glucose these suspensions had no significant oxygen uptake, but with succinate the uptake is considerable.

Bacterial bodies.—These have only a slight effect on the uptake, but the tendency is always towards an increase (Table V).

TABLE V.—*The Effect of the Toxic Fractions and of the Bacterial Bodies on the Oxygen Uptake of Washed Muscle Suspensions in the Presence of Succinate. Final Concentration of the Fraction, 1·0 mg. per c.c.*

		Added substrate.	Oxygen uptake in c.mm. 20 min.	40 min.	60 min.	Percentage effect on oxygen uptake.
1. Muscle alone	(3)	Succinate	57	105	146	
,, + bacterial bodies	(3)	0·4 p.c. Ditto	62	113	159	+ 9
2. ,, alone	(3)	,,	52	98	142	
,, + bacterial bodies	(3)	,,	55	104	152	+ 7
3. ,, alone	(3)	,,	36	61	84	
,, + F68	(3)	,,	5	8	8	— 90
4. ,, alone	(3)	,,	34	64	98	
,, + F68	(3)	,,	3	4	5	— 95
5. ,, alone	(3)	,,	53	100	149	
,, + crude F68	(3)	,,	10	12	12	— 92
6. ,, alone	(3)	,,	48	82	110	
,, + F68/P	(3)	,,	44	73	94	— 15
7. ,, alone	(3)	,,	59	108	153	
,, + F68/P	(3)	,,	44	89	129	— 16

The figures for oxygen uptake are the averaged results of the readings of the number of manometers (shown in brackets) used.

F68.—In a series of experiments (Table V) with different muscle suspensions, F68 in a final concentration of 1·0 mg. per c.c. brought about an inhibition in the oxygen uptake which amounted to between 80 and 95 p.c. Recorded in Chart 3 is an example of results obtained with smaller amounts of the fraction. In this experiment a final concentration of 1·0 mg. per c.c. produced an inhibition of 84 p.c. in two hours and more than half the total uptake was in the first fifteen minutes. Final concentrations of 0·5 and 0·25 mg. per c.c. brought about inhibitions of 67 and 30 p.c. respectively. Smaller concentrations of the fraction did not have any definite effect under the conditions of these experiments, in which the mixtures were made immediately before the beginning of the run. In one experiment with crude F68, this fraction was found to be no less inhibiting than F68 at a concentration of 1·0 mg. per c.c.

F68/P.—This toxic fraction also produced an inhibition of oxygen uptake, but to a very much smaller degree than F68. At a concentration of 1·0 mg. per c.c. the inhibition was only about 15 p.c. (Table V).

F68/D + L and F95.—These non-toxic fractions had only a slight and probably not significant effect on the oxygen uptake of muscle in succinate (Table VI).

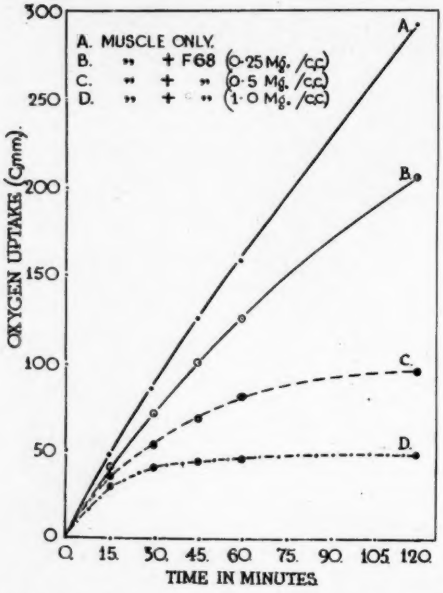

CHART 3.—The effect of F68 in inhibiting the oxygen uptake of washed muscle in 0·4 p.c. succinate. The amount of dry weight of muscle was 40·0 mg. in 1 c.c. of muscle suspension.

TABLE VI.—*The Effect of the Non-Toxic Fractions on the Oxygen Uptake of Washed Muscle Suspensions in the Presence of Succinate. Final Concentration of the Fraction,* 1·0 *mg. per c.c.*

		Added substrate.	Oxygen uptake in c.mm. 20 min.	40 min.	60 min.	Percentage effect on oxygen uptake.
1. Muscle alone	(3)	Succinate 0·4 p.c.	51	97	134	
" + F68/D+L	(3)	Ditto	62	106	144	+ 7
2. " alone	(3)	"	25	36	55	
" + F68/D+L	(3)	"	27	38	54	− 2
3. " alone	(3)	"	47	79	107	
" + F95	(3)	"	44	77	100	− 7
4. " alone	(3)	"	19	35	51	
" + F95	(3)	"	17	35	48	− 6

The figures for oxygen uptake are the averaged results of the readings of the number of manometers (shown in brackets) used.

Experiments on the Effect of Washing Muscle after it has Stood in Contact with F68.

The purpose of this experiment was to determine whether muscle which had been exposed to the action of F68 would recover its capacity for oxygen uptake by washing in water. To 4 c.c. of a washed muscle suspension were added 3 c.c. of a solution of F68 containing 9 mg. of the fraction. To another 4 c.c. of a muscle suspension 3 c.c of water were added. These were allowed to stand for 20 minutes with occasional shaking. The liquids were then centrifuged, the supernatant poured off and the deposits of muscle again washed in about five times their volume of tap-water. This washing was repeated four times. The treated and untreated muscle suspensions were then tested in the Barcroft micro-respirometer in the presence of succinate and buffer. The experimental results are recorded in Table VII. The muscle

TABLE VII.—*The Oxygen Uptake in 0·4 p.c. Succinate of Muscle Washed after Standing in Contact with F68.*

In Experiment 1 the treated suspension was allowed to stand with 9·0 mg. of the fraction, and in Experiment 2 with 6·0 mg. After this the muscle suspension was washed four times with about five times its volume of tap-water.

		Dry weight of muscle in mg.	Oxygen uptake in c.mm.				Percentage difference in oxygen uptake.
			20 min.	40 min.	60 min.	120 min.	
1. Muscle alone	(3)	25·0	24	46	62	114	— 83
,, treated with F68	(3)	22·0	2	6	9	17	
2. ,, alone	(3)	30·0	33	59	83	145	— 48
,, treated with F68	(3)	30·0	20	33	44	76	

The figures for oxygen uptake are the averaged results of the readings of the number of manometers (shown in brackets) used.

treated with the bacterial fraction showed a much smaller oxygen uptake than the untreated muscle, and the difference represented an inhibition of 83 p.c. In a second experiment a slightly larger amount of muscle was exposed to 6·0 mg. of the bacterial fraction and was washed with a larger amount of water. Here the inhibition was 48 p.c. It would appear from these results that some of the bacterial fraction was adsorbed on the surface of the muscle or combined with the succinate enzyme, because if all the bacterial fraction remained soluble in water, the four washings and ultimate dilution would have reduced the final concentration of the fraction to between 0·001 and 0·002 mg. per c.c., and it was shown in the previous experiments that about 0·1 mg. per c.c. was required to produce an effect when tissue and fraction were mixed before the test.

DISCUSSION.

The foregoing experiments have demonstrated that those fractions derived from *Bact. aertrycke* which produce blood-sugar changes and toxic effects on intravenous injection into rabbits, have also the property of increasing *in vitro* the initial oxygen uptake of brain suspensions in the presence of glucose and of inhibiting the respiration of muscle suspensions and of brain washings

in the presence of succinate. This increase in the oxygen uptake of brain appears to be the result of a genuine stimulation, which is not due to F68 acting as a substrate because in the absence of glucose there is little, if any, increase in respiration when the fraction is added. Nor is this increase due to the F68 itself acting as an enzyme, since it was shown to have no demonstrable oxygen uptake in the presence of glucose alone, and, furthermore, the fraction was always added to both cups of the manometer. It is to be noted that F68/P, which appears to be almost as toxic to rabbits as F68, but is very slightly toxic to mice, had a smaller effect than F68 in modifying the oxygen uptake of rabbit tissue suspensions. The intact bacterial bodies, though toxic to the living animal, were without effect in these experiments, and this may be due to their insolubility.

In distinction to the pronounced influence of the toxic fractions on oxygen uptake, the non-toxic fractions had no significant effect on either brain in glucose or washed muscle in succinate. This striking difference between the two groups of bacterial fractions makes it a possibility that similar modifications of tissue respiration play some part in the mechanism of the toxic state in the living animal. On the other hand, it may be that these properties of the toxic bacterial fractions are merely chance effects in the sense that they are without significance *in vivo* because the concentration at which a significant effect on oxygen uptake is produced is much greater than that required to cause toxic effects in the animal. In these experiments the proportion of bacterial fraction which is needed to produce a significant increase in the oxygen uptake of brain, is of the order of one part to two thousand parts of wet weight of brain as compared with the 2·0 to 5·0 mg. dose per kg. of body-weight required to cause severe illness on injection into the living animal. On the other hand, the concentration of the fraction in the blood immediately after injection is relatively great, and, if the brain tissue, which weighs less than 1 p.c. of the total weight of the animal, should have any particular chemical affinity for the fraction, it might be that in experiments on the living animal the concentration of the toxic substance in the brain or in particular parts of it may approach that which is effective *in vitro*. It is clearly impossible at the moment to say whether or not these alterations in the oxygen uptake of brain and muscle also occur in the animal body, but these observations may at some time be of value in helping to elucidate the chemical mechanism of toxic bacterial action.

SUMMARY.

1. Fractions derived from *Bact. aertrycke* which produce blood-sugar changes and toxic effects on injection into rabbits, have the power of greatly increasing the oxygen uptake of rabbit brain suspensions shaken for one to two hours in the presence of glucose and to a lesser extent in the presence of lactate, but have no effect if no substrate is added.

2. These toxic fractions slightly diminish the oxygen uptake of brain *suspensions* in the presence of succinate.

3. These toxic fractions greatly diminish the oxygen uptake of brain *washings* and of washed muscle in the presence of succinate.

4. Non-toxic fractions have no significant effect on the oxygen uptake of brain suspensions in glucose or of washed muscle in succinate.

Our thanks are due to the Medical Research Council for a whole-time grant to one of us (H. A. S.) and for a grant in aid of expenses.

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ANTIBACTERIAL IMMUNITY TO INFECTION WITH HÆMOLYTIC STREPTOCOCCI IN RABBITS.

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Received for publication July 29th, 1936.

MANY workers have described the production of immunity to experimental streptococcal infection in laboratory animals, but the factors upon which such immunity depends are still very imperfectly understood. It would appear, from work by Parish and Okell (1928), and Hartley (1928), that resistance to infection may be produced by inoculation with the bacteria-free toxic filtrate of cultures. While this antitoxic immunity is, so far as it goes, effective against different antigenic types of hæmolytic streptococci, it is by no means complete. Immunized animals are protected against an acutely fatal septicæmic infection, but not from the multiplication of the organisms within the tissues, associated with arthritis, endocarditis and other lesions, resulting in death within a few weeks. Moreover, as Downie (1930) has shown, such an antitoxic immunity is most effective against highly toxigenic strains, with little invasive capacity. Against infection with highly virulent strains, little evidence of protection can be demonstrated. However, by inoculation with heat-killed suspensions of virulent strains, freed as far as possible from toxin by washing, evidence of a more efficient immunity, believed to be mainly antibacterial, has been obtained. This has been shown to be effective against infection with highly virulent strains, provided that they are of the same serological type as that used for immunization. Against heterologous types, however, there is no evidence of protection. Such a

type-specific immunity has been demonstrated by Lancefield and Todd (1928), Downie (1930) and others.

The object of the experiments described here has been to investigate in greater detail the possibility of producing an antibacterial immunity of this type in rabbits; to study the process of immunization at different stages, and the effect of prolonged immunization. Preliminary experiments have also been recorded to demonstrate whether such an immunity might be effective against infection with a strain of heterologous type, and also the possibility of its passive transfer to other animals.

MATERIAL AND METHODS.

The strain "Usher" was used for immunization to infection in nearly all the tests described. It was obtained from Dr. L. Colebrook, and had been isolated from a case of tonsillitis in contact with a fatal case of puerperal septicæmia. When first tested it was found to be moderately virulent for rabbits, the average lethal dose when inoculated intravenously being about 0·1 c.c. of a washed 18-hour Hartley broth culture. Intradermally, it usually failed to kill, even when very large doses were employed. An attempt was made to increase the virulence by passage. After intravenous and intrapleural passage through twenty rabbits, the virulence was greatly increased, the intravenous average lethal dose being then about 0·00001 c.c., or 2000 viable organisms, while intradermally 0·02 c.c. or 4×10^6 viable organisms usually, but not always, sufficed to kill. This passage strain remained viable and virulent for more than three years in rabbit spleen, dried *in vacuo* over phosphorus pentoxide and kept in the ice-chest.

Another strain, L69, was used in the cross-protection experiment. It was obtained from a normal throat and was highly virulent on isolation, the average lethal dose intravenously being 100–1000 viable organisms.

Preparation of vaccine.—The passaged "Usher" strain was inoculated into Hartley broth and grown for 17 hours at 37° C. The organisms were deposited by centrifuging, and resuspended in sterile saline. After washing twice in fresh saline, the organisms were resuspended in saline to 1/5 of the volume of the original broth culture and killed by heat at 56° C. for 40 minutes. The suspension was stored in the ice-chest until required. 1 c.c. was employed as the standard dose for immunization. This appeared to be entirely non-toxic for rabbits.

Immunization of rabbits.—Fifty sandy-lop rabbits, weighing between 1500 and 2500 g. were given weekly intravenous inoculations of vaccine, each receiving 1 c.c. of vaccine per week, over a period extending up to eight months.

TESTS FOR ACTIVE IMMUNITY TO INTRAVENOUS AND INTRADERMAL INFECTION WITH THE HOMOLOGOUS STRAIN.

At intervals, samples from the immunized stock were taken, and with an equal number of normal rabbits as controls, were tested for resistance to infection with the homologous strain. For each test the organism was grown

in Hartley broth for 17 hours. The culture was washed twice in fresh Hartley broth, and dilutions were made in sterile Ringer solution. Batches of rabbits containing two immunized and two normal controls were inoculated intravenously with the diluted living culture, each animal receiving 0·0001 c.c., or approximately 20,000 living organisms. Similar batches of rabbits were depilated on the back and infected intradermally with dilutions varying from 0·004 c.c. to 0·2 c.c. of the washed culture.

The course of infection.—During the week following each test inoculation, daily records were kept of the general condition of each rabbit, including weight and temperature readings. The presence and degree of bacteræmia were recorded by daily counts of the number of organisms in the blood of each animal, by a technique similar to that employed by Wright (1927) and Downie (1930). Samples of blood were taken from the lateral vein of the uninoculated ear and dilutions of each sample were made in sterile Ringer solution. Blood-agar plate cultures were prepared from each dilution and also from undiluted samples. Colony-counts of suitable plates were made 24 hours later and the number of colonies developing from 1 c.c. of sample were estimated. A similar count was made on the inoculum, and the number or organisms per c.c. of blood immediately after inoculation estimated, the total volume of blood being taken as 5 p.c. of the body-weight.

In animals infected intradermally, daily observations on the local lesion were also made. Those surviving were kept under observation for from 28 days to 5 months. They were then killed and autopsy performed. Those dying during the test were examined at once.

TABLE I.—*The Results of Infection with the Homologous Strain at Different Stages of Immunization.*

Intravenous Infection.

Test dose given seven days after.	Infecting test dose.	Vaccinated.		Controls.	
		Survived.	Died.	Survived.	Died.
1st weekly dose of vaccine	0·0001 c.c.	2†	0	0	2
3rd ,, ,, ,,	,,	2	0	0	2
9th ,, ,, ,,	,,	2	0	1†	1
12th ,, ,, ,,	,,	2	0	0	2
20th ,, ,, ,,	,,	2	0	0	2
27th ,, ,, ,,	,,	1	1*	0	2

Intradermal Infection.

1st weekly dose of vaccine	0·004 c.c.	0	2	1	1
3rd ,, ,, ,,	0·02 ,,	1†	1	1†	1
9th ,, ,, ,,	0·2 ,,	2	0	0	2
12th ,, ,, ,,	,, ,,	1	1	0	2
21st ,, ,, ,,	,, ,,	2	0	0	2
29th ,, ,, ,,	,, ,,	2†	0	0	2

† Indicates incomplete resistance in surviving animal, shown by development of arthritis, or other complication.

* Animal injured leg on second day of test and had to be killed.

It will be seen from Table I that immunity has been produced to adequate lethal doses of the infecting strain, given both intravenously and intradermally. The actual stage, however, at which the immunity was optimal was difficult to ascertain, owing to the few animals employed in each test. There appeared to be evidence of resistance to intravenous infection even after one immunizing dose, both the vaccinated animals surviving, though one developed severe arthritis. There was no evidence of increased resistance to intradermal infection at this stage, since both vaccinated animals died, in spite of the fact that the dose administered was insufficient to kill both control animals. After the third immunizing dose, resistance to intravenous infection

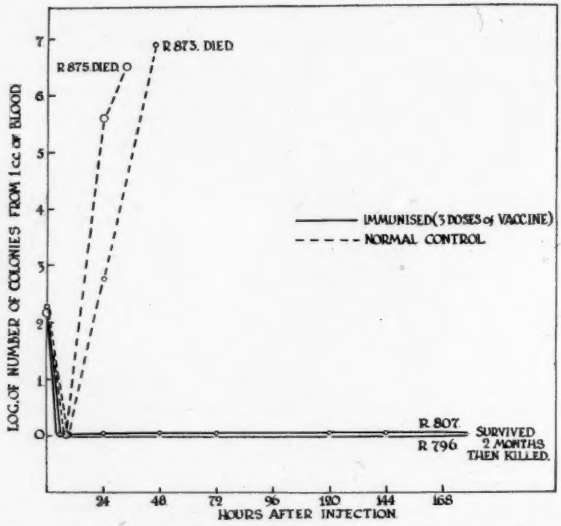

CHART 1.—Bacteræmia following intravenous inoculation of living culture.

was well developed. Both the vaccinated animals survived. There was no bacteræmia after the first few hours and no significant rise in temperature or loss in weight. No complications developed. After survival for two months the post-mortem showed nothing abnormal.

Chart 1 indicates the course of the bacteræmia occurring in these animals. It is essentially similar to those obtained in subsequent intravenous tests in this series. In the immunized animals, after infection, the blood-count shows an immediate and permanent fall. In the control animals, after a temporary fall, there is an immediate rise, culminating in death within forty-eight hours.

In the intradermal test after the third week, the infecting dose, though raised, was still inadequate, one control animal surviving. There was still no evidence of greater resistance in the vaccinated animals. Immunity to intravenous infection would thus appear to have been well developed before there was any immunity to intradermal infection. It should be noted that immunity to the

intradermal inoculation of living streptococci *may* have developed at a much later stage, since no test was made between the third and ninth injection of vaccine. After the ninth immunizing dose further tests were made. Immunity to intravenous infection was still present, but the result was not as clear-cut as in the previous test, as one of the control animals failed to die.

In the intradermal test, however, with a further increase in the infecting dose, both controls died, and both the immunized animals survived.

As is seen in Chart 2 there was an irregular bacteræmia in the immunized animals, disappearing in about a week. In the controls the count gradually rose, culminating in death in about the same period. There was no evidence

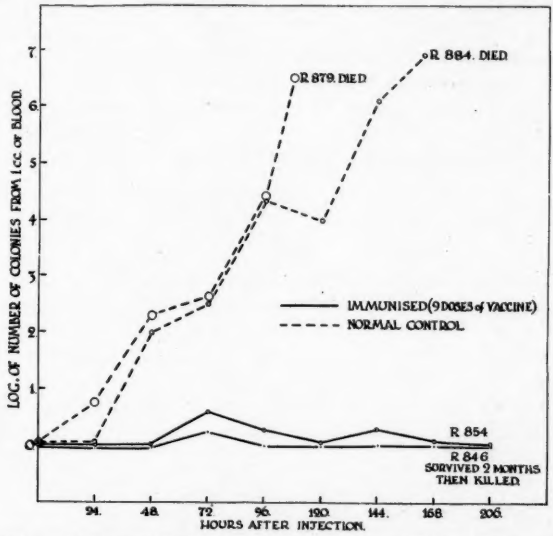

CHART 2.—Bacteræmia following intradermal inoculation of living culture.

of complications in the immunized animals, and on post-mortem two months later nothing abnormal was found, apart from the healing skin-lesion.

At this stage, therefore, resistance to intradermal infection had become more definite.

Further tests were made at intervals up to the 29th week of immunization. Immunity appeared to be maintained throughout the series, against both intravenous and intradermal infection, though there were signs of a decrease in the last intradermal test, when both animals, though surviving, were very ill with severe arthritis. With so small a number of animals this may have been due to chance ; but it may be noted that Topley (1933) has recorded an increase, followed by a decrease in resistance, in mice receiving repeated weekly injections of a vaccine prepared from *Bact. aertrycke*. There is, at least, no evidence in the present experiment that any increase in resistance resulted from prolonging the course of immunization beyond the ninth dose of streptococcal vaccine.

The local lesion in the intradermally infected rabbits.—Both control animals and immunized stock tested at various stages during immunization, developed a very severe local reaction after the inoculation of living culture into the skin. The lesions were similar in character, though slight individual variations occurred, but there was no definite alteration as the result of continued immunization. After twenty-four hours, some superficial necrosis of the skin was present at the site of injection, surrounded by an area of intense erythema varying from 5 to 10 cm. in diameter. The neighbouring blood-vessels were extremely congested. After three or four days necrosis of the skin was much increased, extending over an area 2 or 3 cm. in diameter. There was some spread of the inflammatory process subcutaneously, with induration of the overlying skin. The majority of animals died at this stage. In those immunized animals which survived, however, the localized inflammatory reaction began to subside after the first week. The erythema became less intense, the skin took on a brownish discoloration and desquamation usually followed. The central necrotic area formed a slough and eventually the lesion healed, with considerable scarring of the skin and subcutaneous fibrosis.

PASSIVE IMMUNITY TO INTRAVENOUS INFECTION WITH THE HOMOLOGOUS STRAIN.

An attempt was made to protect normal rabbits passively by the transfer of serum from those actively immunized. Rabbit 862, which had received 27 weekly doses of vaccine, was bled 6 days after the last injection. The serum was injected intravenously into two normal rabbits, each receiving 10 c.c. Two hours later these animals, together with two controls, which received no serum, were inoculated intravenously with a lethal dose of infecting culture, consisting of 9000 viable organisms. The course of infection was followed as in previous tests.

TABLE II.

Hours after infection.	Number of colonies developing from 1 c.c. of blood.			
	Passively immunized.		Normal controls.	
	R. 81.	R. 83.	R. 84.	R. 86.
Immediately	130	130	130	130
24 hours	<1	<1	200,000	Dead 20 hours $>100\times10^6$
48 „	<1	<1	Dead 44 hours $>100\times10^6$	..
72 „	<1	<1	—	—
120 „	<1	<1	—	—

As may be seen from Table II, illustrating the course of the bacteræmia, the immunized animals rapidly cleared the infecting organism from the circulation, while in the controls there was rapid multiplication leading to death within two days.

THE TYPE-SPECIFICITY OF THE IMMUNITY INDUCED.

A preliminary experiment was undertaken to determine whether the immunity induced was effective against infection with a strain of different type. Strain L69, isolated from a normal throat and found to be highly virulent for rabbits was used together with strain "Usher" in this cross-protection experiment. Certain modifications were made in the preparation of the vaccines. The centrifuged and washed deposit from the Hartley broth culture was killed by standing in acetone overnight at 37° C., and was dried off *in vacuo* over phosphorus pentoxide. Batches of six rabbits were immunized with the vaccine prepared from each strain, each animal receiving one dose intravenously per week for four weeks. Each dose consisted of 1 mg. per kg. body-weight of the vaccine resuspended in sterile saline. Seven days after the last immunizing dose, the rabbits were tested for immunity to intravenous infection, three from each batch being infected with the homologous strain, the other three receiving the heterologous strain. The result of this test is set out in Table III.

TABLE III.

		Rabbits immunized with Usher vaccine.	Rabbits immunized with L69 vaccine.	Normal controls.
Infected with "Usher" strain. Dose consisting of 175,000 viable organisms intravenously or 10–100 A.L.D.	Survived	2	1*	0
	Died	1	2	3
Infected with L69 strain. Dose consisting of 200,000 viable organisms, or 100–1000 A.L.D.	Survived	0	2	0
	Died	3	1	3

* Severely ill with persistent bacteræmia for one week, arthritis in several joints, but eventually recovered. The other surviving animals in each batch showed negative blood-counts after 48 hours and developed no visible complications.

The number of animals tested is far too small to warrant any definite conclusion being drawn from this experiment, but there appears to be evidence of immunity to infection with the homologous strain in each case, but little, if any protection against the heterologous strain.

DISCUSSION.

If these results are taken as a whole they would seem to indicate that an effective antibacterial immunity can be developed in the rabbit against a highly invasive strain of hæmolytic streptococcus. This immunity, when fully established, is effective not only against intravenous inoculation, but also against inoculation into the skin, which favours the production of a primary lesion from which the tissues are subsequently invaded. The increased resistance is associated with the appearance of protective antibodies in the blood, as is shown by the passive protection tests. It is type-specific and thus ineffective against antigenically heterologous strains of hæmolytic streptococci.

At the time when this study had reached the stage recorded in this paper, Raistrick and Topley (1934) had succeeded in isolating from *Bact. aertrycke* a partially purified antigenic fraction that induced active immunity in mice. It was, therefore, decided to continue this investigation along similar lines, in collaboration with other workers; and experiments are being carried out on the immunization of mice against virulent strains of hæmolytic streptococci by the inoculation of various chemical fractions isolated from the bacterial cells. The preliminary results obtained in this investigation will be published shortly.

SUMMARY.

1. Immunity to infection with hæmolytic streptococci, believed to be mainly antibacterial, may be developed in rabbits against intravenous and intradermal infection with the homologous strain.

2. There is evidence that immunity to intravenous infection is developed before that to intradermal infection.

3. Preliminary experiments indicate that this immunity may be passively transferred to normal rabbits and that it is not effective against infection with a heterologous strain.

My thanks are due to Dr. L. Colebrook, who kindly supplied the " Usher " strain.

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CENTRIFUGATION STUDIES: I. CRITICAL EXAMINATION OF A NEW METHOD AS APPLIED TO THE SEDIMENTATION OF BACTERIA, BACTERIOPHAGES AND PROTEINS.

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Received for publication August 16th, 1936.

THE method of sedimentation analysis, in which disperse systems are subjected to the influence of intense fields of centrifugal force under conditions for which Stoke's law holds, provides a valuable means of determining the particle size and degree of homogeneity of suspensions. The great potentialities of the method have been revealed during recent years through the technique evolved by Svedberg and his colleagures (1923, 1924 onwards). They have directly analysed the behaviour of sedimenting colloidal and molecularly dispersed systems (protein solutions in particular) by recording periodically measurements of the gradient in optical properties of the system in the direction of the line of action of the applied centrifugal force, which has ranged in magnitude up to one million times gravity. The ultimate purpose of the present study has been the application of centrifugal analysis to suspensions of viruses. However, the conditions for the application of the method in the form developed by Svedberg, namely that the system should lend itself to direct photometric study, either through specific light absorption or refraction, even if the special highly elaborate apparatus were available, cannot be complied with in this case. The amount of virus material ordinarily present is too minute to be measurable by such methods, and recourse must be made to animal inoculation tests. It becomes imperative therefore to devise a technique whereby samples of the system may be withdrawn for test at the end of the period of centrifugation.

The possibility of using the ordinary bucket type of laboratory centrifuge for studying quantitatively the sedimentation of particles of colloidal dimensions has been carefully considered by Bechhold and Schlesinger (1931) and Schlesinger (1934) in studies with different viruses and bacteriophages. According to the technique developed by Schlesinger (1932), the virus suspension is placed in a cylindrical glass tube which is about 5 cm. long and 1 cm. in diameter, and has a flat bottom on which rests a disc of thick filter-paper. The latter, which must be carefully saturated with the liquid at the start of the experiment and freed from all air-bubbles, serves to retain the virus particles as they

sediment to the bottom of the tube, and thus minimizes any tendency to redispersion. After spinning for a given period at a known speed the supernatant liquid is poured from the tube and tested for its content of virus. The relationship between C_o and C_t, the concentrations of virus in the supernatant liquid at the commencement and after time, t, respectively, is derived on the assumption that at any instant during the spinning the supernatant liquid is thoroughly mixed as the result of the inherent mechanical vibration and the convectional disturbances. Then by considering the rate at which particles are removed through traversing the boundary between the liquid and the filter-paper, the particle motion being assumed to follow Stoke's law, the expression deduced is—

$$d = 6{\cdot}15 \times 10^8 \sqrt{\frac{\eta h \log. C_o/C_t}{(\sigma_p - \sigma_m).\ R.T.N.^2}} \quad . \quad . \quad . \quad (1)$$

where d = particle diameter in $m\mu$.
η = viscosity of medium in C.G.S. units.
σ_p = density of particle.
σ_m = density of medium.
h = height of liquid column.
R = distance of filter-paper from axis of rotation.
N = r.p.m.

The validity of the assumptions made has been carefully checked up on a series of gold solutions of known particle size (Schlesinger, 1932).

The estimations of the particle sizes of viruses and bacteriophages, however, based on this equation, although relatively in good agreement, are individually found to be about 30 p.c. higher than the values given by other methods, such as ultraviolet light photography and filtration. The regular occurrence of a discrepancy of this order throughout a series of experiments demands explanation, and it would appear that two factors may contribute towards the rather high figures yielded by the centrifugation method; firstly the mixing in the supernatant may not be quite as complete as assumed, and secondly the density values used for the calculation of the particle sizes may be too low. Evidence upon these points will be discussed later.

The present investigation has aimed at securing conditions of sedimentation under which the disturbing influences are minimized sufficiently to justify the direct application of Stoke's law without further assumptions. The problem has developed in the following sequence: (1) preliminary studies of the factors influencing the degree of sedimentation, (2) the evolution of a technique designed to suppress the disturbing effects, and (3) the application of this technique to, and the critical analysis of the results furnished by suspensions of bacteria, bacteriophages and proteins.

THE CENTRIFUGE.

The centrifuge used in these studies has been an "Ecco" supercentrifuge, by Collatz, of Berlin, capable of speeds up to 15,000 r.p.m. The spindle is an extension of the shaft of a specially built D.C. motor, by which it is thus directly driven. It carries suspension arms for four buckets,

each of which takes a tube of 5 c.c. capacity. These are contained within a hemispherical bowl, which is closed by a flat removable lid held securely in position by a screw cap fitting over the threaded spindle. The air friction during spinning is minimized in this way. The complete centrifuge is built into a strong metal case, and is suspended by adjustable chains from a frame which may be rotated about its single point of attachment to a strong rectangular stand. Access to the spindle of the centrifuge is permitted by a hole in the lid of the metal case. A calibrated rheostat in series with the motor enables the speed to be regulated. The machine attains its speed rapidly, *e. g.* 4000 r.p.m. in less than 1 minute, or 10,000 r.p.m. in a little over 1 minute, while it comes to rest very quickly also, *e. g.* within 1 minute from 4000 r.p.m., or within 1½ minutes from 10,000 r.p.m.

Measurement of speed.—A simple optical method has been used. A cylindrical tubular extension was attached to the screw cap, which fitted the spindle of the centrifuge, projecting for 2 cm. above the top of the metal case. A hole was drilled through this tube at right angles to its axis. Light from a pointolite lamp was gathered by a lens and directed as a narrow beam through the hole in the centrifuge extension tube, on to the periphery of a toothed disc which could be rotated at a controlled speed. The teeth were viewed in a telescope set in alignment with the beam of light. Suppose the centrifuge to be spinning at N r.p.m.; then as the speed of the rotating disc is increased, the *first* stationary image of the teeth seen in the telescope will occur when a tooth is just replaced in its position by the one following in the time interval between successive axial passages of the light through the hole in the centrifuge extension. This condition is given by $2N = x\,S$, where x = number of teeth, and S = speed of the disc in r.p.m. The speed of the disc was regulated by means of a potentiometer resistance in series with a small universal motor, and was measured by counting the revolutions by the aid of a speedometer and stop-watch. In order to embrace the complete range of speeds up to 15,000 r.p.m. two discs each having 72 teeth were coupled by gears in the ratio 1 : 7·5.

This method enables the speed to be measured without affecting the centrifuge in any way, and is therefore to be preferred to the use of a tachometer.

RELATIONSHIP GOVERNING IDEAL SEDIMENTATION.

The velocity of sedimentation, v, of a small spherical particle having density σ_p and radius r, in a liquid medium of density σ_m, and viscosity η under the influence of a centrifugal force is given by the relationship :

$$v = \frac{2r^2\,(\sigma_p - \sigma_m)\,w^2\,x}{9\eta} \quad . \quad . \quad . \quad . \quad (2)$$

where w = angular velocity; x = distance of particle from the centre of rotation.

The assumptions implied for the validity of this relationship are :

(*a*) That the particle is spherical in shape.
(*b*) That the motion of the particle is uniform.
(*c*) That the particle is moving freely in the liquid unhampered by neighbouring particles.

With these conditions obtaining, if the particle moves from x_1 to x_2 (distances measured from the axis of rotation) in time t then :

$$\int_0^t r^2\ dt = \int_{x_1}^{x_2} \frac{9\eta}{2\ (\sigma_p - \sigma_m) w^2} \cdot \frac{dx}{x}$$

$$\text{whence } r = \sqrt{\frac{9\eta\ ln\ x_2/x_1}{2(\sigma_p - \sigma_m)\,w^2\,t}} \quad . \quad . \quad . \quad . \quad (3)$$

It is therefore possible in the case of a monodisperse system settling with a clearly defined boundary to evaluate r from measurements of x_1 and x_2, knowing the densities σ_p and σ_m, the value of t and the speed of the centrifuge. Clearly, however, it is very necessary to ensure that the experimental conditions comply with the requirements demanded for the validity of the relationship expressed in equation 2.

DISTURBING INFLUENCES ENCOUNTERED IN THE CENTRIFUGATION OF SUSPENSIONS.

(1) *Convection effects.*—Variations in temperature between different parts of the liquid will give rise to convection currents and so prevent uniform motion of the sedimenting particles. Ordinarily a temperature gradient may be expected to develop between the liquid next the walls of the centrifuge tube and that in the centre, owing to the heat generated during spinning through air friction, and possibly some conduction of heat from the motor. The air friction is minimized in the present apparatus by having the buckets enclosed in a hemispherical bowl as already described. The liquid to be centrifuged has always been brought to a temperature of 20° to 22° C. at the beginning of the experiment, and with ice-cold water circulating in the cooling jacket the final temperature has seldom exceeded 25° to 26° C. The following table shows how the temperature of water varied with the duration of centrifugation at 10,000 r.p.m:

TABLE I.—*Temperature Changes during Centrifugation of Water contained in Metal Buckets.*

Duration of run in hours.	Temperature (°C.)
..	20·2
0·25	22·3
0·50	23·7
1·00	24·7
2·00	25·4
3·00	25·8

Volume of water in bucket = 3 c.c. Diameter of bucket = 1·8 cm.

With the liquid contained in glass tubes within the metal buckets the temperature is somewhat lower.

The ice-cold water jacket may be expected to maintain the gradient of temperature so that the coolest regions, and therefore the densest portions of the liquid, are those most removed from the axis of rotation—conditions under which convectional disturbances should be greatly mimimized.

(2) *Mechanical vibration.*—The centrifuge used in these experiments has run in a surprisingly steady manner, and so long as the speed is constant, the vibration must be small. However, as with most centrifuges, noticeable

vibration occurs during the processes of starting and stopping. There appears to be a zone of resonance vibration in the region of 8000 r.p.m. Externally applied vibration will produce its greatest effect at points in the system where a phase density difference exists, *e. g.* at the liquid/air interface or at the surface of a layer of powdered solid in a liquid. In the former instance a disturbance will originate in the liquid surface and be transmitted into the bulk of the liquid, becoming damped as it proceeds. Should it reach the bottom of the tube, it will be reflected and tend to redisperse any sedimented particles. Again, the mechanical vibration may tend directly to stir up a sediment if this is not tightly packed. However, in practice such redispersion of sedimented particles may be prevented by having at the bottom of the centrifuge tube something to which the particles may adhere, viz. a layer of agar or gelatin gel, or in which they may be held by entanglement, as happens when a disc of thick filter-paper is used (*cf.* Schlesinger, 1932). The magnitude of the mixing effect in the liquid column will depend upon the violence of the causative impulses, and upon the extent of free surface by way of which the disturbance may be transmitted. This is well borne out in experiment, for sedimentation is found to be much more efficient in capillary tubes of 1 to 2 mm. diameter than in wide tubes of 1 to 2 cm. diameter. Further, when the liquid is contained in a closed cell the effects of vibration become negligible, since it is extremely difficult to produce mixing within a self-contained volume of fluid by shaking the system.

(3) *Technique of sampling.*—Since with the bucket type of centrifuge it is necessary to sample the system at the end of a run, it is essential that this operation should be made without serious mixing of the liquid. Satisfactory sampling by hand is quite impossible. Experiments were made in which sampling was effected by means of a capillary pipette with the tip bent at right angles. This was carefully lowered into and raised out of the liquid by having it mounted on a rack-and-pinion movement, while the taking of the liquid into the capillary was by means of a rubber teat controlled with a screw clip. Results obtained by this technique were quite reproducible, and the evidence showed that a concentration gradient existed in the liquid column, although no defined boundary was detected. The difference in concentration at different heights could be most readily demonstrated in narrow tubes, and was not very marked in wide tubes. Thus the assumption made by Bechhold and Schlesinger, namely that mixing is complete, would appear to be justified for wide tubes, the conditions they actually select.

Experiments were also made in which bacterial suspensions were centrifuged in capillary tubes, which, at the end of the run, were broken at points etched by means of a diamond. Here also the concentration gradient was demonstrated.

A NEW TECHNIQUE FOR STUDYING THE SEDIMENTATION OF SUSPENSIONS WITH THE ORDINARY BUCKET TYPE OF CENTRIFUGE.

A device incorporating the advantages of having a cell of narrow diameter and no free liquid meniscus has provided a method of studying sedimentation

under conditions in which most of the disturbing factors discussed in the previous section are very considerably reduced. The arrangement is shown in Fig. 1, where (*a*) illustrates the early design. A small cylindrical glass cell, 0·5 cm. or less in diameter and 1 cm. in height, prepared by fusing a piece of glass tubing to the end of a glass rod of the same external diameter, is carried in a metal adaptor collar which just fits the centrifuge bucket. This collar is of suitable diameter at its lower end to fit within the flat-bottomed centrifuge tube carried in the bucket. The inverted glass cell, previously filled with liquid, dips into a measured volume of liquid in the glass centrifuge tube, so that the outside liquid level corresponds with the closed end of the inverted cell. A later simplified design is shown in Fig. 1 (*b*). This is an all-metal cell made of M.V.C. alloy, an aluminium-silicon alloy prepared by Metropolitan Vickers Electrical Co. Ltd. The alloy is possessed of great tensile strength, 10 tons per sq. inch.,

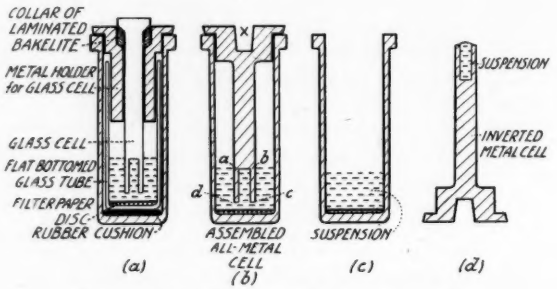

FIG. 1.—Centrifuge cells, design and assembly.

is light, having a density value 2·68 (*cf.* aluminium, 2·7), and is readily machined. It has the further valuable property of being noncorrodible in salt water, and it may be sterilized repeatedly at 160° to 180° C. in the hot-air oven without any detrimental effect. Tests carried out with bacteria, bacteriophages and viruses suspended in broth at pH 7·6 have in no instance revealed any deleterious action after 6 hours of contact with the metal. However, certain purified bacteriophages when suspended in saline have been found to lose upwards of 99 p.c. of their activity after 6 hours' contact with the metal. In all cases, therefore, the action of the metal should be carefully controlled, for the toxic effects of inorganic metallic ions vary enormously, and what may suit one phage may inactivate another (see Burnet and McKie, 1930). Broth appears to exert a protective influence. In studying suspensions of purified bacteriophages and viruses it is advisable to make the measurements in glass cells. Apart from this the advantages of the all-metal cell are obvious. The dimensions of the components can be accurately machined, and the inverted cell with the adaptor collar can be combined in one unit (Fig. 1 (*d*)), while the centrifuge bucket forms the other (Fig. 1 (*c*)). In filling the cell a calculated volume of the liquid is first measured into the bucket, and then the inverted cell is filled separately as in Fig. 1 (*c*) and (*d*). A disc of thick filter-paper is

placed at the bottom of the bucket to retain any deposit during centrifugation. Care must be taken to saturate the disc thoroughly and remove all air-bubbles. The bucket and inverted cell with their contents are then poised to within a milligram against the opposite pair, and the complete assembling is very simply effected by combining the components as in Fig. 1 (*b*). Should it be necessary to adopt a rigorous aseptic technique the two portions may be assembled immediately after filling, and the accurate counterpoising be done by adding drops of a suitable non-volatile liquid to the cup *x*, Fig. 1 (*b*).

That portion of the system contained within the inverted cylindrical cell *abcd*, Fig. 1 (*b*), may be considered as a self-contained volume, from which sedimentation may occur through the open end *cd*. The absence of free surface eliminates certain vibrational disturbances as well as any "meniscus effect" due to surface adsorption and evaporation during long runs. The advantage of having a narrow tube is also gained in the inverted cell, and the fact that the latter dips into the solution restricts the free surface of the outside liquid, thus acting as a baffle to any surface vibration that may originate there. Difficulties of sampling are largely removed by a cell of this design. Immediately the centrifuge has stopped the inverted cell is lifted directly from the bucket, the volume *abcd* remaining filled with liquid through the forces of atmospheric pressure and capillarity. The convexity of the surface at *cd* varies according to the surface tension of the solution and the precise manner in which the cell is withdrawn. The procedure adopted has been to take a coarse capillary, and then by lightly touching the lip—the cell standing as in Fig. 1 (*d*)—to remove the excess liquid. Touching the surface momentarily with filter-paper can serve the same purpose. The complete contents of the cell are then withdrawn for analysis, and a comparison made with the unspun solution.

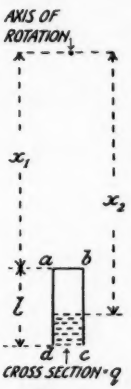

FIG. 2.

Theoretical.—If C_o and C_t are the concentrations of the centrifugable constituent contained in the liquid filling the cell at the beginning and end of the run respectively, let us consider how these are related assuming Stoke's law to be applicable.

Let *abcd* in Fig. 2 represent the cell, *ab* being at distance x_1 from the axis of rotation.

Height of cell = 1, and the cross-section = q. Considering that a homodisperse system fills the cell at the commencement, then if the boundary of the particles falls from x_1 to a level at distance x_2 from the axis in time t, it may be shown that—

$$\frac{x_2}{x_1} = e^{Kt}, \text{ where } K = \frac{2}{9}\frac{r^2(\sigma_p - \sigma_m)w^2}{\eta}.$$

Therefore: Height of clear liquid $= x_2 - x_1$

$$= x_1(e^{Kt} - 1).$$

If the velocity of sedimentation were uniform, the concentration c_T in the layer of suspension still contained within the cell would be equal to C_o, but this is not so, since the acceleration changes with the distance from the axis of rotation. We find that—

$$c_T = C_o \quad e^{-Kt}.$$

The average concentration of particles C_t contained within the volume of the cell at the end of time, t, is therefore given by—

$$C_t = \frac{C_o\, e^{-Kt}\, q\, [l-(x_2 - x_1)]}{q\, l}.$$

Whence—

$$\frac{C_t}{C_o} = \frac{e^{-Kt}\, (l + x_1) - x_1}{l}.$$

From this the following relationship is deduced :

$$r^2 = \frac{9\eta\, ln \left\{ \frac{x_1 + l}{x_1 + l\, C_t/C_o} \right\}}{2\, (\sigma_p - \sigma_m)\, w^2\, t}.$$

$$r = \sqrt{\frac{9\eta\ 2{\cdot}303\ \log. \left\{ \frac{x_1 + l}{x_1 + l\, C_t/C_o} \right\}}{2(\sigma_p - \sigma_m)\, w^2\, t}} \quad \text{All in C.G.S. units.}$$

Whence—

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta \log. X}{(\sigma_p - \sigma_m)\, N^2\, t}} \quad (4),$$

where d = particle diameter in mμ. ; $X = \frac{x_1 + l}{x_1 + l\, C_t/C_o}$; N = r.p.m. ; t = time in mins.

It should be emphasized that this relationship is valid only as long as the conditions implied in Stoke's law prevail. As already indicated, in practice there are invariably influences tending to cause departure from the ideal conditions postulated. The following sections deal with the experimental studies of the new inverted capillary tube technique as applied to typical suspensions of bacteria, bacteriophages and proteins.

APPLICATION OF THE NEW TECHNIQUE.

Systems Studied and Methods of Analysis.

(a) *B. prodigiosus.*—Suspensions in the specified media of 48-hours-old cultures grown on 2 p.c. nutrient agar at 22° C. have been used.

Concentrations were determined by counting the colonies developing on agar plates sown with 0·1 or 0·05 c.c. amounts of known dilutions. In all tests at least 3 plates were used for each estimation, and in special cases the mean of counts made on 6 plates was taken. (This also applies to the phage estimations.)

(b) *Bacteriophages Staph. K. and S.* 13.—These are respectively the anti-staphylococcus and anti-salmonella phages studied by Elford and Andrewes (1932), who, by ultrafiltration analysis, found Staph. K phage (50 to 75 mμ.) to be one of the largest and S 13 (8 to 12 mμ.) to be the smallest of the bacteriophages then investigated. Staph. K phage inoculated into young broth cultures of the susceptible *Staph. aureus* has invariably effected complete lysis after 2 hours' incubation at 37° C. A 0·7μ membrane filtrate of the lysed culture usually contains about 10^9 particles per c.c. The plaques normally produced by Staph K phage are extremely small; with 1 p.c. agar they are only 0·1 mm. in diameter. This renders their accurate counting very difficult. The following procedure resulting in the formation of larger plaques largely overcomes this difficulty. The 1 p.c. agar plates spread with the phage and susceptible organism are dried and then incubated for 2 hours at 37° C. The plates are then withdrawn from the 37° C. incubator and placed at 22° C. overnight. The preliminary incubation enables both the organism and phage to multiply, but during the 12 hours at 22° C. the multiplication is retarded, while the diffusion of the phage already generated may proceed so that an extended radius of action results in the formation of larger plaques. Reincubation for an hour at 37° C. accelerates multiplication again, and the final clearly defined plaques, 0·5 mm. diameter, are readily counted.

The S 13 phage has been propagated in broth cultures of a rough variant of the Flexner dysentery strain Y6 (National Collection of Type Cultures). Complete lysis was never produced, but a 0·7μ membrane filtrate prepared after 6 hours' incubation of the young inoculated culture contained 10^7 to 10^8 particles per c.c., as indicated by plaque counts on 2 p.c. agar.

(c) *Proteins: hæmocyanin (Helix), edestin and egg albumin.*—Solutions of crystallized preparations of these proteins were used; viz. hæmocyanin by the method of Svedberg and Chirnoaga (1928), edestin by Osborne's method (1909), and egg albumin after Hopkins and Pinkus (1898). The concentrations of solutions were determined refractometrically with the Zeiss dipping refractometer, and also checked by nitrogen estimations by the micro-Kjeldahl method.

Dimensions of Cells.

Cells 1 cm. in length and 0·5 cm. or 0·25 cm. in diameter have been used in these studies. Evidence confirming the fact that sedimentation is the more complete the narrower the cell was early obtained, and is well illustrated by the data of Table II. Therefore cells 0·25 cm. or less in diameter are preferable. In order that more liquid might be available for testing than is furnished by a single cell of these dimensions, it was arranged to have seven such cells dipping simultaneously into the fluid in each bucket. This was effected by drilling the cylindrical metal cell as shown in Fig. 3. The seven

parallel cells provided 0·3 c.c. of liquid for test. It is often desirable in experiments with viruses to have at least 0·5 c.c. of fluid available, and this could be assured by using a pair of the 7 (0·20 × 1·0 cm.) cells.

In describing the cells used in particular experiments in the tables the letters M. and G. have been chosen to indicate metal and glass respectively, while the figure immediately following denotes the diameter. Thus G. 0·25 means a glass cell 0·25 cm. in diameter.

The distance x_1 of the upper closed end of the cell from the centre of rotation is 7·25 cm. for the metal cells and 6·75 cm. for the glass cells. All

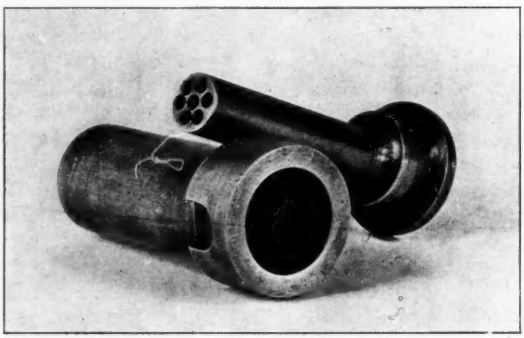

FIG. 3.

cells have the same length, namely 1 cm. In the tables $\log. X = \log. \left(\frac{x_1 + l}{x_1 + l\ C_t/C_o}\right)$. Therefore for the metal cells $\log. X = \log. \frac{8{\cdot}25}{7{\cdot}25 + C_t/C_o}$, and for the glass cells $\log. X = \log. \frac{7{\cdot}75}{6{\cdot}75 + C_t/C_o}$.

Influence of the Time of Centrifugation.

The theoretical relationship of equation (4) governing normal sedimentation in a cell such as that used in these experiments implies that under otherwise constant conditions the time of centrifugation t and $\log. X$, where $X = \frac{x_1 + l}{x_1 + l\ C_t/C_o}$, should be directly proportional to each other. Finally, when $C_t = 0$ any further increase of t cannot alter the value of $\log. X$. Experimental determinations were made of C_t for different values of t to ascertain how far the actual sedimentation obeyed the above theoretical requirements. Table II contains data for *B. prodigiosus*, staph. K phage and hæmocyanin, while the corresponding graphs of $\log. X$ against t are given in Fig. 4. Examination of the latter shows that initially there is an approximately linear relationship between $\log. X$ and t, but as the value of C_t/C_o approaches 0·1, the linearity fails and the slope falls off, $\log. X$ reaching asymptotically its limiting value.

TABLE II.—*Influence of Time on Degree of Sedimentation.*

Suspension.	Medium.	Cell.	N (r.p.m.).	t (mins.).	$C_{o'}$	$C_t/C_{o'}$	Log. X.
B. prodigiosus	Water.	M. 0·5	4,500	5	$2{\cdot}8\times10^6$/c.c.	0·107	0·0498
			„	10	„	0·006	0·0558
			„	15	„	0·003	0·0560
			5,000	15	$2{\cdot}0\times10^6$/c.c.	0·0025	„
			„	30	„	0·0022	0·0561
			„	60	„	0·002	„
			„	120	„	0·0013	„
	„	M. 0·25	4,300	2·5	$3{\cdot}5\times10^4$/c.c.	0·37	0·0345
			„	5	„	0·086	0·0510
			„	10	„	0·008	0·0557
			4,250	15	$9{\cdot}5\times10^3$/c.c.	0·005	0·0559
Staph. K phage	Broth pH 7·6	M. 0·5	11,000	15	$5{\cdot}7\times10^4$/c.c.	0·632	0·0199
			„	30	„	0·351	0·0356
			„	60	„	0·114	0·0494
			„	120	„	0·019	0·0551
		M. 0·25	„	30	$4{\cdot}2\times10^4$/c.c.	0·262	0·0408
			„	60	„	0·057	0·0528
			10,750	120	$3{\cdot}0\times10^3$/c.c.	0·009	0·0557
Hæmocyanin	*M*/15 phosphate pH 6·81	M. 0·5	11,100	30	0·22%	0·79	0·0112
			„	60	„	0·59	0·0222
			„	90	„	0·41	0·0323
			„	120	„	0·35	0·0357
	M/15 phosphate pH 7·31	M. 0·25	11,000	30	0·10%	0·76	0·0129
			„	60	„	0·61	0·0211
			„	120	„	0·41	0·0323
			„	180	„	0·23	0·0426

Thus when C_t/C_o is less than 0·1, *i. e.* while the sedimentating boundary is definitely contained within the cell, the demands of equation (4) may be said to be satisfied. As the boundary approaches the bottom of the cell the departure from normal sedimentation becomes significant, and the disturbing forces present in the outer liquid, largely non-existent within the confines of the cell, make their influences felt to an increasing extent. Therefore the theoretical relationship can with justification be used for calculating particle sizes only when the values of C_t/C_o are not greater than 0·1. The method is thus only just applicable to virus suspensions, for the reliable detection of less

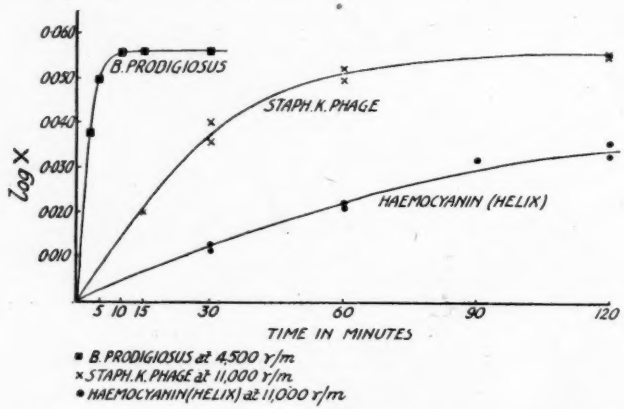

FIG. 4.—Curves showing how log X varies with the time of spinning.

than tenfold differences in concentrations by means of the animal inoculation test is seldom possible. However, for systems where more accurate methods of measuring concentration are available, *e. g.* phages by the plaque count method, and proteins by refractometric analysis, the range of application is correspondingly greater.

Estimation of Particle Density by Centrifugation.

In calculating the radius of a particle from sedimentation data it is necessary to know its density in relation to that of the medium (see equation (4)). According to Stoke's law the force acting on a small spherical solid particle of density σ_p in a liquid of density σ_m is proportional to $(\sigma_p - \sigma_m)$. Thus when $\sigma_p = \sigma_m$ the particle will remain stationary, and hence by making observations on the behaviour of the particle in media of different known densities the value of σ_p may be determined. While this is a straightforward method for macroscopic solid particles, difficulties arise in applying it in the cases of microscopic and ultramicroscopic particles, even when powerful centrifugal forces are available. Thus as the density of the medium is varied by increasing the concentration

of suitable solute, *e. g.* sucrose, altered osmotic equilibria may be expected to modify the size and density of the dispersed particles. Further, in the case of sugar solutions in particular, the viscosity increases rapidly with concentration, and the consequent retardation in the rate of sedimentation adds to the difficulty of determining the true condition for zero movement. Other factors of practical importance for the success of the method are the influence of the medium on the viability of bacteria, viruses and bacteriophages, and also the degree of accuracy with which slight differences in concentration of the suspension may be determined. If measurements of the rate of sedimentation are made for two values of σ_m, then it is possible to evaluate σ_p from the two simultaneous equations. However, the necessity for an accurate means of determining small differences in concentration presents a difficulty in applying this method to viruses.

Another procedure is possible with turbid suspensions, namely carefully to superimpose a layer of the concentrated suspension above the sugar solution of known density, and then after spinning for a given time to observe directly whether or not the particles have entered into the lower layer. This method would minimize any influence the sugar solution might have on the viability of micro-organisms. The possibilities of this method are being investigated.

The results obtained during the present study illustrating the effect on sedimentation produced by changes in the density of the medium will be discussed in the sections dealing with the particular systems concerned.

Centrifugation of B. prodigiosus.

In order to secure those conditions most favourable for the reliable measurement of the rate of sedimentation of *B. prodigiosus*, *i. e.* so that the ratio C_t/C_o is less than 0·1, it has been necessary to run the centrifuge at low speeds, *e. g.* at 4000 r.p.m. for periods not longer than 5 minutes, and at 2000 r.p.m. for periods up to 15 minutes.

Density.—Centrifugation of *B. prodigiosus* suspended in aqueous sugar solutions has shown that when the density of the medium increases from 1·0 to 1·1, the value of C_t/C_o changes from 0·002 to 0·85 for otherwise comparable spinning conditions. In view of our knowledge of the morphology of bacteria, it may be expected that estimations of the densities of bacteria suspended in solutions of high solute content will be relatively high owing to loss of water by the cell through osmotic influences. Direct evidence that this does occur has been furnished in the recent work of Ruffilli (1933), who found that while the normal density of *B. coli* was 1·094, its value after a short period of contact at room temperature with 10 p.c. sugar solution was 1·102, and for 18 p.c. sugar was 1·111. The corresponding densities of the organism after 24 hours' contact with the solutions at 0° C. were 1·111 and 1·136 respectively. Thus if the period of contact is kept short then the effect is quite small. In the present series of experiments the time of contact did not exceed two hours, and the indicated density of *B. prodigiosus* was $1{\cdot}10 \pm 0{\cdot}02$*. Other estimations of the density of this organism to be found in the literature are 1·054 by

* The sedimentation data for the organism suspended in Ringer and broth media given in Table III indicate a value 1·11 for the density of *B. prodigiosus*.

Rubner (1890) using a pyknometric method, and figures 1·17 to 1·245 for cultures 40 and 80 days old, and therefore not comparable with those used here, by Stiegell (1908), who observed the rise or fall of massed groups of the organism in solutions of potassium carbonate of different densities. Recently Kossowski (1936), by determining the weight and volume of cells packed in a calibrated capillary, has found a value of 1·109.

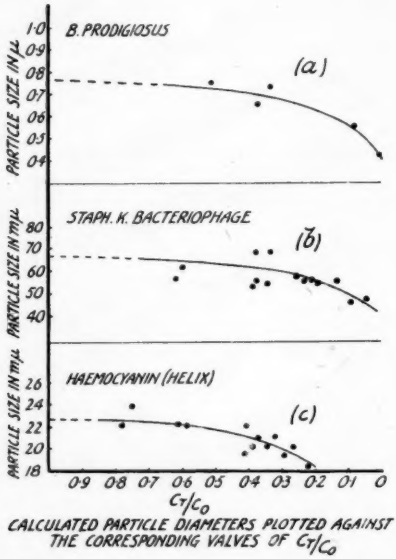

FIG. 5.—Calculated particle diameters plotted against the corresponding values of Ct/Co.

Particle size.—It will be seen in Table III that the calculated values of the particle diameter, d, decrease rapidly as the ratio C_t/C_o becomes smaller than 0·1. This effect is primarily the result of mixing as the sedimenting boundary approaches the bottom of the cell, but the fact that the suspension is not truly homodisperse may be a contributory factor too. The values of d are plotted against C_t/C_o in Fig. 5 (*a*). Extrapolating back graphically for conditions corresponding to small displacements of the sedimenting boundary (this portion of the curve cannot be explored reliably by experiment, owing to the limitations of the accuracy of the method of estimating the concentration of organisms by colony counts), the particle size of *B. prodigiosus* is indicated to be 0·7 to 0·8μ. This is in complete agreement with the average diameter of this organism measured directly under the microscope, viz. 0·75μ, and may be regarded as providing indirect confirmation of the density value 1·10 employed in these calculations.

TABLE III.—*Centrifugation Data for* B. prodigiosus.

Medium.	Density (σ_m).	Viscosity (η).	Cell.	N (r.p.m.).	t (mins.).	C_o.	C_t.	C_t/C_o.	Log. X.	Particle diameter (d) in microns.	Remarks.
Water	0·997	0·0090	M. 0·2	4,300	2·5	$3·5 \times 10^4$	$1·3 \times 10^4$	0·37	0·0345	0·64	Density of *B. prodigiosus* used in calculating d in all cases, 1·10.
					5·0	„	$3·0 \times 10^3$	0·086	0·0510	0·55	
					10·0	„	$3·0 \times 10^2$	0·008	0·0557	0·41	
„	„	„	„	2,500	15·0	$1·75 \times 10^5$	$1·4 \times 10^4$	0·08	0·0514	0·55	
	„	„	G. 0·2	2,000	10·0	$2·8 \times 10^6$	$2·2 \times 10^6$	0·79	0·0119	0·81	
Ringer	1·003	0·0093	M. 0·2	4,000	2·5	$4·8 \times 10^4$	$1·7 \times 10^4$	0·354	0·0355	0·73	
Broth	1·018	0·0107	„	„	„	$5·5 \times 10^4$	$2·8 \times 10^4$	0·509	0·0267	0·74	

Centrifugation of Bacteriophages.

Several workers have demonstrated the possibility of spinning down bacteriophages by the use of high centrifugal forces (D'Herelle, 1921 ; Gratia, 1934 ; Girard and Sertic, 1935 ; McIntosh, 1935), but the work of Schlesinger (1933) appears to have been the only attempt prior to the present study at measuring the rate of sedimentation of bacteriophages with the view to estimating their particle sizes. Schlesinger's results will be compared later with the figures obtained in the experiments about to be described.

I. *Staph. K. bacteriophage.*—Upwards of 90 p.c. of this phage is removed from the inverted cell when centrifuged for one hour at 11,000 r.p.m. (see Table IV, in which the results obtained with Staph. K. phage are summarized). This ready sedimentation renders possible the estimation of the density of the bacteriophage particles by studying the rate of settling in sugar solutions of measured densities and viscosities. The data in the third group of experiments in Table IV indicate that sedimentation of the phage is arrested in a medium of density 1·25. The viscosities of the systems rise rapidly for densities exceeding 1·20, and this itself exerts a retarding influence on the sedimentation, tending to obscure the true conditions for zero movement corresponding to $\sigma_p = \sigma_m$. The apparent density, therefore, 1·25, may yet be slightly low, in spite of the opposing tendency of osmotic influences.

The particle diameters given in the table have been calculated on the assumption that 1·25 is the density of the phage, and have been plotted against the corresponding values of C_t/C_o in Fig. 5 (*b*). Here extrapolation of the curve for conditions corresponding to small displacements of the sedimenting boundary indicates the size of the Staph. K phage particles to be 60 to 70 mμ. It is interesting to compare this value with determinations made according to the method of Schlesinger (see Table V). Six experiments yielded figures for the particle diameter of this phage ranging from 62 to 78mμ. The two methods provide results which are in excellent agreement.

II. *S.* 13 *bacteriophage.*—This phage sediments less readily than Staph. K phage, centrifugation for 3 hours at 11,000 r.p.m. being barely sufficient to reduce the concentration of a broth suspension to 50 p.c. of the original (see Table VI). Thus a reliable estimation of density by the method of zero displacement is not practicable. Also it is doubtful whether viruses of the same order of size as S. 13 phage, like those of foot-and-mouth disease and poliomyelitis, can be considered as coming within the scope of the present method.

In the absence of direct experimental evidence regarding the density of S. 13 phage, the same value as found for Staph. K phage, namely 1·25, has been used in calculating the particle diameters given in Table VI. The results indicate the size of S. 13 phage to be 15 to 17 mμ., compared with the value 8 to 12 mμ. determined by ultrafiltration analysis (Elford and Andrewes, 1932). The Experiment 3 in Table VI was conducted on a preparation of purified S 13 phage which had been washed with saline on a suitable membrane until protein could no longer be detected. The figures obtained for the particle size of this preparation of phage when suspended in saline are definitely higher

TABLE IV.—*Centrifugation Data for Staph. K Phage.*

Experiment.	Medium.	Density (σ_m.)	Viscosity (η).	Cell.	N (r.p.m.).	t (mins.).	C_o.	C_t.	C_t/C_o.	Log. X.	Particle diameter (d) (mμ.).	Remarks.
1	Broth pH 7·6	1·014	0·0109	M. 0·5	11,000	15	$5·7 \times 10^4$	$3·6 \times 10^4$	0·632	0·0199	56·5	σ_m and η values at 25° C.
						30	„	$2·0 \times 10^4$	0·351	0·0356	53·5	Density of phage=1·25.
						60	„	$6·5 \times 10^3$	0·114	0·0494	44·5	
						120	„	$1·1 \times 10^3$	0·019	0·0551	33	
				M. 0·25	„	30	$4·2 \times 10^4$	$1·1 \times 10^4$	0·262	0·0408	57	
						60	„	$2·4 \times 10^3$	0·057	0·0528	46	
					10,750	120	$3·0 \times 10^5$	$2·8 \times 10^3$	0·009	0·0557	34	
				G. 0·3	11,100	15	$5·4 \times 10^6$	$3·2 \times 10^6$	0·593	0·0234	61	
						30	„	$2·1 \times 10^6$	0·389	0·0357	52	
2	Broth-Ringer	1·005	0·0092	M. 0·5	11,000	30	$1·8 \times 10^6$	$3·5 \times 10^5$	0·194	0·0447	54	..
					„	60	„	$7·8 \times 10^4$	0·043	0·0536	42	
	1 : 100 pH 7·4	1·007	0·0093	M. 0·25	11,200	15	$1·05 \times 10^7$	$3·4 \times 10^6$	0·331	0·0367	69	
						30	„	$1·5 \times 10^6$	0·143	0·0477	55	
				G. 0·3	11,100	15	$6·5 \times 10^6$	$2·4 \times 10^6$	0·369	0·0369	69	
						30	„	$1·4 \times 10^6$	0·215	0·0464	55	
3	Broth	1·012	0·0104	M. 0·5	10,800	180	$2·2 \times 10^6$	$3·5 \times 10^3$	..	..	..	All sugar solutions were made in broth.
	30% sugar	1·165	0·0455	„	11,500	165	$2·6 \times 10^5$	$9·0 \times 10^4$	..	..	..	
	„	„	„	„	12,500	120	$4·5 \times 10^5$	$1·1 \times 10^5$	..	..	..	
	40% „	1·187	0·0631	„	11,500	165	$2·6 \times 10^5$	$9·5 \times 10^4$	..	..	..	
	45% „	1·222	0·0795	„	„	„	$2·5 \times 10^5$	$1·5 \times 10^5$	..	..	..	
	50% „	1·246	0·1191	„	„	„	„	$2·6 \times 10^5$	..	..	..	
	„ „	„	„	„	10,750	120	$2·3 \times 10^5$	$2·2 \times 10^5$	..	..	..	
	„ „	1·247	0·1195	„	10,500	135	$2·5 \times 10^5$	$2·4 \times 10^5$	..	..	..	
	60% „	1·302	0·5775	„	„	„	$3·3 \times 10^5$	$3·5 \times 10^5$	..	..	..	
	„ „	„	„	„	„	„	$4·0 \times 10^4$	$4·5 \times 10^4$	..	..	..	
4	Broth	1·014	0·0109	G. 0·3	11,000	30	$1·45 \times 10^6$	$5·6 \times 10^5$	0·386	0·0358	54	This experiment was made with phage which had been washed free of bacterial protein on a suitable membrane.
	Broth-Ringer 1 : 100	1·005	0·0092	„	„	„	$3·7 \times 10^6$	$8·5 \times 10^5$	0·230	0·0454	55	

TABLE V.—*Particle Size of Staph. K Phage by Method of Bechhold and Schlesinger.*

Medium.	σ_m.	η.	N (r.p.m.).	C_o.	C_t.	C_t/C_o.	h (cm.).	R (cm.).	Time (mins.).	Particle diameter (d) ($m\mu$).
Broth-Ringer 1 : 100	1·006	0·0094	10,500	$1·0 \times 10^6$	$2·0 \times 10^4$	50	1·00	8·0	120	62
Broth	1·012	0·0104	10,800	$2·2 \times 10^6$	$5·0 \times 10^3$	440	1·25	„	180	74
„	„	„	10,000	$4·0 \times 10^4$	$6·8 \times 10^3$	6	1·20	„	60	73
„	„	„	11,600	$4·4 \times 10^4$	$1·2 \times 10^4$	3·7	1·25	„	30	78
„	„	„	„	„	$5·5 \times 10^3$	8	„	„	60	69
„	„	„	„	„	$1·0 \times 10^3$	44	„	„	100	72

TABLE VI.—*Centrifugation Data for* S 13 *Phage.*

Experiment.	Medium.	σ_m.	η.	Cell.	N (r.p.m.).	t (mins.).	C_o.	C_t.	C_t/C_o.	Log. X.	Particle diameter (d) ($m\mu$).	Remarks.
1	Broth pH 7·6	1·012	0·0104	M. 0·5	11,500	180	$4·0 \times 10^4$	$2·2 \times 10^4$	0·55	0·0244	17	Density of phage = 1·25.
				„	„	120	$3·0 \times 10^4$	„	0·73	0·0145	16	
				„	11,100	„	$2·5 \times 10^4$	$1·85 \times 10^4$	0·74	0·0140	„	
				„	12,250	150	$1·2 \times 10^4$	$7·0 \times 10^3$	0·59	0·0222	„	
				M. 0·25	11,500	120	$3·0 \times 10^4$	$2·1 \times 10^4$	0·70	0·0161	17	
2	Broth pH 7·6	1·014	0·0103	M. 0·5	12,250	150	$1·75 \times 10^4$	$1·2 \times 10^4$	0·69	0·0167	15	..
				„	10,800	120	$7·0 \times 10^3$	$5·4 \times 10^3$	0·78	0·0118	16	
				M. 0·25	12,500	180	$1·1 \times 10^5$	$5·8 \times 10^4$	0·53	0·0255	17	
3	Broth pH 7·6	1·014	0·0109	„	11,200	190	$5·5 \times 10^4$	$3·0 \times 10^4$	0·55	0·0244	„	The phage used in Expt. 3 had been purified by washing it on a $25m\mu$. membrane with saline as medium. It was diluted 1 : 100 in broth and saline respectively for the experiments.
	Saline pH 6·65	1·005	0·0094	G. 0·2	„	180	$4·7 \times 10^4$	$6·5 \times 10^3$	0·14	0·0511	23	
		„	„	G. 0·3	„	„	„	$5·4 \times 10^3$	0·12	0·0523	24	

than when broth is the medium, suggesting that the phage may be partially aggregated in the former liquid. This supports the conclusion previously drawn from the results of filtration studies with purified bacteriophages (Elford and Andrewes, 1932).

Three experiments made according to the method of Schlesinger yielded the values 20, 15 and 12 mμ. for the particle diameter of S13 phage, the density being taken as 1·25. The erratic figures are doubtless due to the inaccuracy of the method of estimating the small differences in the concentration of the phage such as are obtained with liquid columns 1 cm. in depth and speeds of 11,000 r.p.m. for periods of 2 to 3 hours. The size of S13 phage found by Schlesinger (1933) was 20 mμ. based on a density of the phage of 1·12 (personal communication from Dr. Schlesinger). Recalculated for the density 1·25 as used in the present study Schlesinger's figure becomes 14 mμ.

Centrifugation of Proteins.

I. *Hæmocyanin.*—Svedberg and Chirnoaga (1928) and Svedberg and Heyroth (1929) have found that hæmocyanin (*Helix*) when in dilute buffer solutions behaves on centrifuging as a monodisperse suspension within the limits of pH 4·5 to 7·6. The particles were found to be spherical in shape, and their calculated diameter was 24mμ. for the partial specific volume 0·735. Solutions of this protein therefore constitute an excellent test system for the present method. The results which have been obtained are given in Table VII. The values of the particle diameters are based on the value 1·36 for the density of hæmocyanin, *i. e.* the reciprocal of the partial specific volume quoted above.* The calculated diameters are plotted against the corresponding values of C_t/C_o in Fig. 5 (*c*). The extensive series of measurements indicate the particle size of hæmocyanin (*Helix*) in aqueous solution to be 22 mμ.—a figure in good agreement with the value 24 mμ. found by Svedberg and his collaborators.

In Table VIII the results of a series of experiments according to the technique of Schlesinger are given. The mean of five determinations gives 20 mμ. as being the particle diameter of the protein in solution.

The data of Table VII lead to the further conclusions (1) that the particle size of the protein is not affected by changes of pH within the range 4·5 to 7·2 (Expt. 2), (2) that for otherwise similar conditions the protein in solutions ranging in concentrations from 0·1 to 1·4 p.c. shows a slightly enhanced rate of sedimentation in the more concentrated systems, and (3) that the calculated particle sizes agree most closely with the value 24 mμ., as determined by Svedberg *et al.*, when the conditions correspond to small displacements of the sedimenting boundary.

II. *Edestin.*—This protein has also been studied in the Upsala Laboratory by Svedberg and Stamm (1929), who found that in solutions having concentrations greater than 0·1 p.c. edestin shows normal sedimentation on the alkaline

* It would be preferable to check this value directly in the centrifuge, but the conditions available here are not such as to make this practicable.

TABLE VII.—*Centrifugation Results with Hæmocyanin.*

Experiment.	Medium.	σ_m.	η.	Cell.	N (r.p.m.).	t (mins.).	Concentration protein (%).	C_t/C_o.	Log. X.	Particle diameter (d) (mμ.).
1	*M*/30 phosphate pH 6·2	1·002	0·0090	G. 0·5	10,500	120	1·41	0·38	0·0362	21
				M. 0·5	„	„	..	0·33	0·0368	„
2	*M*/25 acetate „ 4·5	1·001	„	„	11,800	„	0·23	0·30	0·0386	19
	M/15 phosphate „ 6·0	1·003	0·0092	„	„	„	„	0·31	0·0380	„
	„ „ „ 7·2	„	„	„	„	„	„	0·30	0·0386	„
	M/30 „ „ 6·2	1·002	0·0091	„	„	„	0·70	0·27	0·0403	20
3	*M*/15 „ „ 7·2	1·003	0·0092	M. 0·5	11,000	„	0·45	0·35	0·0357	„
4	„ „ „ 7·2	„	„	G. 0·4	11,100	„	„	0·38	0·0362	„
				G. 0·3	„	„	„	0·35	0·0380	„
5	„ „ „ 6·86	„	„	M. 0·5	10,800	„	0·18	0·39	0·0334	„
6	„ „ „ 6·81	„	„	„	11,100	30	0·22	0·79	0·0112	22
				„	„	60	„	0·59	0·0222	„
				„	„	90	„	0·41	0·0323	„
				„	„	120	„	0·35	0·0357	20
7	*M*/30 „ „ 6·8	1·002	0·0090	G. 0·35	11,000	„	0·16	0·40	0·0350	„
	„ „ „ „	„	„	M. 0·25	„	„	„	0·32	0·0374	„
8	*M*/15 „ „ 7·31	1·003	0·0092	„	11,000	30	0·10	0·76	0·0129	24
				„	„	60	„	0·61	0·0211	22
				„	„	120	„	0·41	0·0323	19
				„	„	180	„	0·23	0·0426	18

TABLE VIII.—*Data for Hæmocyanin using the Method of Bechhold and Schlesinger.*

Medium.	σ_m.	η.	N (r.p.m.).	Time (mins.).	Concentration hæmocyanin (%).	C_t/C_o.	h.	R.	Particle diameter (d) (mμ.).
M/20 phosphate buffer	1·002	0·0090	11,000	120	0·16	1·79	1·10	7·5	20
M/15 „ „	1·003	0·0092	11,100	„	0·45	1·50	1·25	8·0	17
M/15 „ „	„	„	11,000	„	„	1·85	1·30	8·5	21
M/15 „ „	„	„	11,800	„	0·23	1·70	„	„	20
M/30 „ „	1·002	0·0090	10,500	„	1·40	1·91	„	„	23

side of its iso-electric point (pH 5·5) up to pH's approaching 11·0, in which region dissociation occurs. On the acid side of the isoelectric point the protein is unstable. The normal molecular weight of edestin was found to be 212,000, the partial specific volume 0·734 giving the density 1·36, and the indicated particle diameter is 8 mμ.

In the present study 5 experiments with crystallized edestin dissolved in 1·5 *M* NaCl in concentrations ranging from 0·5 to 0·9 p.c., and at pH 6·4, have shown after centrifugation for 3 hours at 11,000 r.p.m. an average drop of 10 p.c. in the concentration of protein within the inverted cell. The calculated values of the particle diameter based on the density value 1·36 have ranged from 6 to 10 mμ. The average figure, 8 mμ., is identical with that found by Svedberg and Stamm, but the agreement may be fortuitous, since with centrifugal forces of the magnitude employed here the effects of diffusion would be expected to affect sedimentation and result in the apparent particle size being too low. It may be that this effect is counterbalanced by an abnormally high degree of sedimentation due to the presence of a small percentage of an aggregated form of the protein. Edestin in solution is notorious for its tendency to aggregate, and the presence of quite a small percentage of polymolecular particles would suffice to give an apparently high rate of sedimentation under the conditions producing such small differences in concentration as those measured in the present experimen s.

III. *Egg Albumin.*—No change could be detected in the concentration of a 0·6 p.c. solution of crystallized egg albumin in *M*/15 phosphate buffer after centrifugation in the inverted cell for 3 hours at 11,000 r.p.m.

DISCUSSION.

The value of the inverted capillary tube technique in providing conditions of sedimentation for which Stoke's law may be directly applied has been amply illustrated in the foregoing studies. Provided the system lends itself to reasonably accurate analysis, then by studying the influence of time on the degree of sedimentation it is possible to ascertain the region in which the concentration varies in accordance with Stoke's law. The necessity of being able to estimate small differences of concentration presents a real difficulty when suspensions of viruses are being considered. The method of animal inoculation does not permit the detection of less than tenfold differences in the concentration of virus, except perhaps in certain cases where skin reaction titrations are possible. This means that viruses only just come within the scope of the new inverted tube method in its present form ; for this, it has been pointed out, the lowest value of the ratio C_t/C_o yielding reliable figures for the particle size is 0·1.

The alternative method due to Bechhold and Schlesinger is very simple, and, as applied to studies with viruses, has the advantage that concentrations in the supernatant amounting to one-tenth, one-hundredth, or even one-thousandth of the original may be used in calculating the particle diameter. The assumption of complete mixing made in deriving the relationship governing

the sedimentation in wide tubes appears to be justified; excellent agreement is shown between the calculated particle sizes based on this assumption and those obtained by the new method developed here.

The observations on the density of Staph. K phage are of much significance. The ascertained value 1·25 is definitely greater than the densities of bacteria 1·05 to 1·15; and also higher than the figure obtained by Schlesinger (1932) for the density of anti-coli phage " WLL ", viz. 1·14 (calculated from measurements of the rate of sedimentation of the phage in two media of different known densities), a value which, in view of later experiments, Schlesinger (1935) concluded was somewhat low. The uncertain factor in determining densities by the method of zero displacement is the magnitude of the dehydration occasioned by the high solute concentrations which perforce have to be used in attaining the requisite density values in the medium. It is clear that extended measurements of the densities of bacteria, viruses, bacteriophages and proteins are required, for in addition to assisting in the interpretation of sedimentation data, the figures should provide valuable evidence as to whether viruses and bacteriophages bear a greater resemblance in their make-up to the cellular form of bacteria having a high water content and consequent low specific gravity (1·05 to 1·15), or to protein substance alone, which is of relatively high density (1·30 to 1·35).

The particle size values established with the inverted capillary tube technique compare very favourably with figures given by alternative methods. Thus *B. prodigiosus* is here found to be 0·7 to 0·8μ in diameter, while microscopical measurement shows that a suspension of this organism from a 48-hour agar culture may contain forms ranging in size from 0·5 to 1·0 μ, so that the centrifugation method gives a representative average figure. In the case of Staph. K phage the value 60 to 70 mμ. for the particle diameter confirms the finding of Elford and Andrewes (1932), who by ultrafiltration analysis determined the size of this phage to be 50 to 75 mμ. Staph. K phage has also been found to have the same size as C16 phage (Burnet), which from microscopical examination has been estimated to be " probably not much more than 50 mμ." (Burnet, 1933), and which Schlesinger (1933) has included in his group of largest phages having particle diameters of 90 mμ. This latter figure was calculated from centrifugation data on the assumption that 1·10 is the density of the phage, but becomes 57 mμ. if the density is 1·25. However, the possibilities that different types of bacteriophages may have slightly different densities must not be overlooked. The diameter found for S13 phage, 15 to 17 mμ., is higher than that yielded by filtration, 8 to 12 mμ. (Elford and Andrewes, 1932). Using the technique of Schlesinger I obtained the value 13 to 20 mμ., while previously Schlesinger (1933) found the diameter of this phage (assumed density 1·12) to be 20 mμ., or 14 mμ. when recalculated for the density 1·25. The evidence of centrifugation therefore indicates the size of S13 phage to be about 15 mμ. The higher values obtained by Schlesinger for the sizes of several bacteriophages by centrifugation compared with the results of filtration are completely explained by the low value he used for the densities of the phages. The results of the two methods agree well in the light of the new evidence regarding the densities of phages revealed in the present studies.

That the calculated particle sizes of hæmocyanin (*Helix*) and edestin obtained by the inverted capillary tube method should correspond so well with the figures furnished by the more exact and elaborate method of ultracentrifugal analysis of Svedberg is very gratifying, and in itself a confirmation of the new technique. The lower limit in the range of application of the inverted tube method with the centrifugal forces here available is represented by edestin with particles of 8 mμ. diameter for systems having densities comparable with proteins and amenable to exact analysis. In the case of viruses the limit in terms of particle size is probably about 40 mμ., and for bacteriophages about 15mμ.

The current studies, considered together with those of Elford and Ferry (1935) on the comparative filtration of the serum proteins, edestin, and hæmocyanin (*Helix*), systems investigated also by ultracentrifugal analysis by Svedberg *et al.*, have, by demonstrating the good agreement between the results of the centrifugation and filtration methods, greatly consolidated our knowledge of the particle sizes of viruses and bacteriophages acquired during recent years mainly through the application of ultrafiltration analysis.

SUMMARY.

A new technique has been described enabling the sedimentation of bacteria, bacteriophages, viruses and proteins to be studied with the ordinary bucket type of high-speed centrifuge. By arranging for an inverted capillary tube (1 cm. in length and 0·25 cm. or less in diameter) to dip into the main bulk of liquid contained in the centrifuge bucket, sedimentation may occur within the inverted cell under conditions for which Stoke's law may be directly applied. The arrangement minimizes " mixing " and simplifies the process of sampling, since the cell on being withdrawn from the liquid retains the sample to be tested. The particle size of the suspension may be calculated from measurements of the average concentration within the cell at the beginning and end of a period of controlled centrifugation by use of a relationship based solely on Stoke's law.

A critical study of the method has been made by the analysis of data experimentally established for *B. prodigiosus*, Staph. K phage, anti-dysentery phage S13, and the proteins hæmocyanin (*Helix pomatia*) and edestin.

The density of *B. prodigiosus* has been determined as 1·10 and that of Staph. K bacteriophage as 1·25. The available evidence suggests that the bacteriophages have densities approaching those of the proteins, indicating a compact structure in contrast to the cellular morphology of bacteria which have high specific water contents and correspondingly low densities, 1·05 to 1·15.

The following particle sizes have been determined by means of the new inverted capillary tube technique of centrifugation: (1) *B. prodigiosus* 0·7 to 0·8 μ; (2) Staph. K phage 60 to 70 mμ.; (3) S13 phage 15 to 17 mμ.; (4) hæmocyanin (*Helix*) 22 mμ.; and (5) edestin 8 mμ. These values agree well with those obtained by alternative methods, and in particular furnish confirmation of the results of ultrafiltration analysis.

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CENTRIFUGATION STUDIES: II. THE VIRUSES OF VACCINIA, INFLUENZA AND ROUS SARCOMA.

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Received for publication August 16th, 1936.

THE new inverted capillary tube method of studying the rate of sedimentation of disperse systems described in the previous paper (Elford, 1936) has been applied to the viruses of vaccinia, influenza and Rous sarcoma, with a view to comparing the particle sizes of the infective agents given by this method with the figures previously obtained by ultrafiltration analysis (Elford and Andrewes, 1932; Elford, Andrewes and Tang, 1936; Elford and Andrewes, 1935).

The technique of the centrifugation method has been exactly as described in the paper by Elford (1936) for bacteria, bacteriophages and proteins, and will not be detailed here. The experimental objective when working with

virus systems is determined (*a*) by the fact that the method of estimating the concentration of virus by the animal inoculation test does not readily permit the detection of smaller than tenfold differences, and (*b*) by the finding that reliable values for the particle sizes of suspensions are only yielded by the present method as long as the drop in the average concentration within the inverted cell after spinning is not more than tenfold. The aim in each set of experiments therefore has been to establish those conditions—particularly the speed and time of centrifugation—which, in the case of each virus, just suffice to ensure a diminution of the virus concentration to one-tenth of the original. Then the particle diameter of the virus may be calculated from the relationship (for derivation see Elford, 1936)—

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta \log. \dfrac{x_1 + l}{x_1 + l\, C_t/C_o}}{(\sigma_p - \sigma_m)\, N^2\, t}},$$

where d = particle diameter in mμ.
x_1 = distance of top of capillary from centre of rotation = 7·25 cm. for metal cell or 6·75 cm. for glass cell.
l = depth of inverted capillary = 1 cm.
σ_p = density of particle ; σ_m = density of medium ; η = viscosity of medium.
N = speed of centrifuge in r.p.m.
t = time of centrifugation in mins.
C_o = initial concentration ; C_t = concentration after spinning for time t.

For conditions such that $C_t/C_o = 0{\cdot}1$ this relationship becomes—

$$d = k \sqrt{\frac{\eta}{(\sigma_p - \sigma_m)\, N^2\, t}},$$

where $k = 1{\cdot}78 \times 10^7$ for the metal cells or $1{\cdot}84 \times 10^7$ for the glass cells.

VACCINIA.

The virus studied was a neurovaccinia strain. Stock filtrates of emulsions of infected rabbit testes in broth-Ringer mixtures were prepared as described earlier (Elford and Andrewes, 1932). In testing for the amount of virus present serial tenfold dilutions of virus were inoculated intradermally, in 0·1 c.c. quantities, into the skins of duplicate rabbits.

The experimental results are given in Tables I and II. The preliminary experiments at once showed that vaccinia virus could be thrown down very readily, and that for the purposes of analysis by the inverted capillary tube technique a speed of 5000 r.p.m. would be quite sufficient. In addition to experiments on the sedimentation of the virus as contained in stock bacteria-free filtrates of 0·25 p.c. testis emulsions in broth, a further series were made with virus which had been washed free of demonstrable protein so as to provide

TABLE I.—*Centrifugation Results with Vaccinia Virus.*

Date.	Medium.	Density of medium (σ_m).	Viscosity (η).	Speed (r.p.m.).	Time (mins.).	C_o.	C_l.	C_l/C_o.	Remarks.
19.xi.34	Broth-Ringer 1 : 100	1·004	0·0093	10,000	95	10^{-3}, 10^{-2}	Und. neg.	Less than 0·001	Fresh virus, washed on 0·18μ membrane in broth-Ringer.
26.xi.34	„	„	„	„	30	10^{-2}	„	Less than 0·01	Cold-stored virus, washed on 0·18μ membrane in broth-Ringer.
					60	„	„	Ditto	
					90	„	„	„	
3.xii.34	Ringer	1·003	0·0092	7,500	15	10^{-5}	10^{-3}, 10^{-2}	0·001–0·01	Fresh virus, washed on 0·11μ membrane in Ringer.
					30	„	10^{-2}, 10^{-2}	0·001	
10.xii.34	„	„	„	4,750	30	10^{-4}, 10^{-3}	„	0·01–0·1	Cold-stored virus, washed on 0·11μ membrane in Ringer.
					60	„	und. neg.	0·0001	
17.xii.34	Broth-Ringer	1·004	0·0093	4,600	30	10^{-4}, 10^{-4}	10^{-2}, 10^{-2}	0·01	Cold-stored virus, washed on a 0·11μ membrane in broth-Ringer.
					60	„	10^{-2}, 10^{-1}	0·01–0·001	
14.ii.35	Broth	1·014	0·0105	4,500	30	10^{-2}	Und.	0·01	Fresh virus, washed on 0·11μ membranes in broth.
				„	„	„	10^{-1}	0·1	
				„	„	„	„	„	Glass cells used.
				„	„	„	„	„	
28.ii.35	„	1·011	0·0102	4,750	30	10^{-2}, 10^{-3}	10^{-1}, 10^{-1}	0·1–0·01	Stock filtrate of virus in broth used. The σ_m and η values refer to ultrafiltrate through a 0·2 μ membrane.
					60	„	„	„	
7.iii.35	„	1·013	0·0103	4,250	30	10^{-3}, 10^{-3}	10^{-2}, 10^{-2}	0·1	Stock filtrate in broth used; σ_m and η refer to virus-free ultrafiltrate.
14.ii.35	„	1·014	0·0105	4,400	„	10^{-2}, 10^{-2}	10^{-2}, 10^{-1}	Barely 0·1	Stock filtrate in broth diluted 1 : 100 in broth of known density and viscosity.

The virus concentrations are expressed in terms of the limiting reacting dilution. Tests are usually made on duplicate rabbits.
Except in Experiment of 14.ii.35 metal cells were used in all experiments.

TABLE II.—*Centrifugation of Vaccinia Virus in Media of Different Densities.*

Date.	Density of medium.	Viscosity.	Speed (r.p.m.).	Time (mins.).	C_o.	C_t/C_o.	Remarks.
14.xii.24	0·999	0·0092	9,500	60	10^{-4}	0·0001–0·001	Washed virus diluted appropriately in Ringer—sugar solutions of measured densities and viscosities.
	1·056	0·0140	„	„	„	0·01–0·1	
	1·114	0·0238	„	„	„	0·01–1	
	1·173	0·0501	„	„	10^{-3}	1	
8.i.35	1·014	0·0107	10,200	120	10^{-4}	Less than 0·0001	Washed virus diluted in broth—sugar solutions of known densities and viscosities.
	1·147	0·0363	„	„	„	0·01	
	1·180	0·0538	„	„	10^{-3}	0·1–1	
17.i.35	1·149	0·0360	9,750	„	10^{-2}	0·1	Washed virus suspended in broth—sugar solutions of known densities and viscosities.
	1·178	0·0526	„	„	„	1	
24.i.25	1·182	0·0540	10,000	12	„	1	Washed virus suspended in broth—sugar solutions of known densities and viscosities.
	1·220	0·0958	„	„	„	„	

a relatively pure suspension of vaccinia in the chosen medium of known density and viscosity. No difference was detected in the sedimentation behaviour of washed as compared with unwashed virus. The results in Table I indicate that with the virus contained in broth medium a speed of 4500 r.p.m. maintained for 30 mins. produces a drop in the average concentration within the inverted cell to one-tenth the initial value, *i. e.* $C_t/C_o = 0{\cdot}1$.

The ready sedimentation of vaccinia virus makes possible the determination of the density of the virus particles by gradually increasing the density of the medium until movement is arrested. In Table II the result of experiments in which sugar solutions in broth of known densities and viscosities were used as media are summarized. When the medium had a density 1·18 the concentration of virus in the cell remained apparently unaltered after 2 hours' spinning at 10,000 r.p.m. It was calculated that if the true density of the virus were 1·20, then under the existing conditions with density 1·18 and viscosity 0·054, the sedimentation would not be detected, but were the true density 1·25, then a drop in concentration capable of being detected by the method of test here used would be expected. Hence the conclusion was drawn that the density of vaccinia could be expressed as $1{\cdot}18 \pm 0{\cdot}02$. This also makes some allowance for possible osmotic influences. MacCallum and Oppenheimer (1922) found that vaccinia virus was not deposited from glycerine solution of density 1·14, but the absence of particulars of the exact conditions of centrifugation renders impossible any discussion of their result in relation to the higher figure obtained here.

The particle size of vaccinia virus may now be calculated from the sedimentation data already quoted for a suspension in broth. In this case we have

$$d = 1{\cdot}78 \times 10^7 \sqrt{\frac{0{\cdot}0105}{(1{\cdot}18 - 1{\cdot}014)\,(4500)^2\,30}}$$
$$= 182\ \mathrm{m}\mu.$$

When the virus is contained in Ringer solution the rate of sedimentation is more rapid than in broth, and the conditions giving the ratio $C_t/C_o = 0{\cdot}1$ have not been directly ascertained. However the result of the experiment 10.xii.24 in Table I, where C_t/C_o was between 0·1 and 0·01, indicates a particle diameter of 170 mμ.—a figure which should be regarded as a minimum value. The combined evidence of the experiments giving the size of vaccinia virus to be 170–180 mμ. is in good agreement with previous estimations. For instance Barnard (1935) has found the particle diameter to be 150–170 mμ. by ultra-violet light photography; Elford and Andrewes (1932) by ultra-filtration found 125–175 mμ.; while Bechhold and Schlesinger (1931) assumed the density of the virus to be 1·10, and calculated from their centrifugation data that the particle diameter of vaccinia virus was 210–230 mμ., a value which, when corrected for the density value 1·18, becomes 160 to 170 mμ.

INFLUENZA VIRUS.

The virus studied was the W.S. strain adapted to mice (*cf.* Andrewes, Laidlaw and Smith, 1934). 5 p.c. emulsions of infected mouse lung were filtered, after preliminary clarification through asbestos, through 0·6μ membranes to give bacteria-free stock filtrates. The filtrates were diluted 1 : 9 in broth saline for the centrifugation experiments. Falling tenfold dilutions of virus were tested on groups of 3 mice; each mouse received 0·05 c.c. intranasally under ether anæsthesia. The mice were killed 5 days later and their lungs examined for the typical lesions. The highest dilution of a fluid which would produce lung lesions in one or more mice in a 0·05 c.c. dose was taken as the titre.

Early experiments with the broth-saline suspensions of the virus showed that after spinning for 3 hours at 11,000 r.p.m. the concentration of virus in the inverted cell was reduced to one ten-thousandth of the original, *i. e.* $C_t/C_o = 0{\cdot}0001$. The possibility was thus afforded of estimating the density of the virus particles by measuring the sedimentation of the virus in broth-sugar solutions of known densities. After 3 hours' centrifugation at 10,000 r.p.m. in one of two experiments with a density of the medium 1·17 a tenfold drop in concentration was observed, while in three experiments with the density 1·20 no difference between the centrifuged and control sugar systems could be detected. At densities below 1·17 the supernatant liquids were in all cases less active than the controls. There appears to be still some sedimentation at 1·17, but the magnitude of the alteration in the concentration within the cell under the prevailing conditions of centrifugation is barely capable of being detected and is not greater than tenfold. The results are considered to indicate a value for the density of the influenza virus close to 1·20, which figure has been used in calculating the particle diameter.

The experimental results summarized in Table III show that conditions ensuring a tenfold diminution in the concentration within the inverted cell are provided by a speed of 11,000 r.p.m. for 15 mins., and at 4750 r.p.m. for 90 mins., when the virus is contained in a medium of density 1·003 and viscosity 0·0096. The corresponding values for the particle size of the virus

TABLE III.—*Centrifugation Data for Influenza Virus.*

Speed (r.p.m.).	Time (mins.).	C_o.	C_t/C_o.	Remarks.
11,000	180	10^{-5}	Less than 0·0001	In each experiment a stock membrane filtrate of the virus was diluted 1 : 9 in broth—saline having density 1·003 and viscosity 0·0096. All experiments were conducted with the metal cells. The concentration of virus is expressed in terms of the limiting infective tenfold dilution.
„	90	10^{-4}	„ 0·001	
„	15	„	0·1	
„	„	„	0·1–1	
„	30	„	0·01	
5,500	120	„	„	
4,650	90	„	0·1	

(density 1·20) are calculated to be 99 and 87 mμ. The diameter of influenza virus was estimated by ultrafiltration (Elford, Andrewes and Tang, 1936) to be 80–120 mμ.

ROUS SARCOMA VIRUS.

Bacteria-free filtrates were prepared from 2·5 p.c. emulsions of tumour tissue in broth, and the virus was then washed on a suitable membrane, usually of porosity 65 mμ., until free of protein as indicated by a negative sulphosalicylic acid test. This washing treatment is particularly necessary, since the stock filtrates contain appreciable amounts of mucin, and this may be filtered off through membranes which retain the virus. The medium used for washing the virus was broth-Ringer (1 : 10) in all experiments.

Reference should be made to the earlier paper on the ultrafiltration of Rous sarcoma virus (Elford and Andrewes, 1935) for particulars of the procedure followed in estimating the relative virus titres of various liquids. Tests were made with serial tenfold dilutions of the fluids, the rates of growth and final sizes of the tumours being compared.

The results obtained in a series of experiments with washed preparations of Rous sarcoma virus are summarized in Table IV. After 3 hours' spinning at 12,000 r.p.m. the concentration of virus in the inverted cell is reduced to less than 1 p.c. of the original. This degree of sedimentation is not high enough to permit a reliable estimation of the density of the particles by the method of zero displacement in sugar solutions. It has only been possible to prove that the density of the virus is greater than 1·15. The data in Table IV indicate that with unwashed virus contained in the stock filtrate a tenfold drop in the concentration within the cell is effected by spinning for 90 mins. at 11,000 r.p.m., while a slightly greater drop in obtained with the washed virus under the same conditions of centrifugation (see Expt. 26.iii.35). The difference between the rates of sedimentation in the two instances is due to the different viscosities of the media. This is well illustrated in the control tests made with Staph. K bacteriophage, which was added to the system along with the virus. The rate of sedimentation of this phage is seen to be approximately that of the virus, and if similar densities be assumed for both (1·25), then when allowance is made for the disparity in sensitivity of the respective methods for estimating their concentrations, Staph. K phage and Rous sarcoma virus may be said to be very similar in size. The size of Staph. K phage has

TABLE IV.—*Centrifugation Data for Rous Sarcoma Virus.*

Date.	System.	σ_m.	η.	Speed (r.p.m.).	Time (mins.).	Titre of virus.	C_t/C_o.	Remarks.
16.xi.34	Washed virus in Ringer solution	1·003	0·0092	12,000	105	10^{-2}	Less than 0·01	Metal cell was used in every experiment.
26.iii.35	15 c.c. washed virus + 5 c.c. Staph. phage	1·009	0·0100	11,000	90	10^{-3} (phage $2·0 \times 10^5$/c.c.)	Less than 0·1 (phage 0·02)	Titre of virus = limiting infective tenfold dilution.
	15 c.c. unwashed virus + 5 c.c. Staph. phage	1·010	0·0317	„	„	Ditto	0·1 (phage 0·05)	Titre of phage = number of particles per c.c. estimated by the method of plaque-count.
8.v.25	Washed virus + Staph. phage in broth—Ringer	1·008	0·0099	12,000	210	4×10^{-2} (phage 2×10^4/c.c.)	Less than 0·01 (phage 0·005)	
22.v.35	Washed virus in broth—Ringer	„	„	11,000	90	10^{-2}	0·1	
3.vi.35	Washed virus in broth	1·010	0·0104	„	210	10^{-3}	Less than 0·01	
5.vii.35	Washed virus in broth—Ringer	1·006	0·0093	„	90	10^{-1}–10^{-3}	Less than 0·1	

been determined to be 60–70 mμ. (Elford, 1936) by the same method of centrifugation used here. Elford and Andrewes (1935) from the results of ultra-filtration experiments concluded that the Rous sarcoma virus might be as small as 75 mμ., and Yaoi and Nakahara (1935) have also estimated the size of the virus to be 70–105 mμ. The indications of the present study, with due allowances for the uncertainty regarding the density of the virus and the inaccurate means of estimating the virus concentration, support these conclusions.

DISCUSSION.

Several workers have previously demonstrated the possibility of centrifuging down the elementary bodies of the larger viruses, and used it as a means of freeing the virus from much of the associated tissue material. Of the viruses studied here vaccinia has been so treated by Bland (1929), Craigie (1932), and Eagles and Ledingham (1932), and Rous sarcoma virus by Ledingham and Gye (1935) and McIntosh (1936). However, the work of Bechhold and Schlesinger (1931), to which reference has already been made, represents the only attempt, before the present studies, to apply the centrifugal method for obtaining figures for the particle sizes of viruses. The technique as described by Schlesinger (1932) has been discussed in relation to the present method in the earlier paper by Elford (1936). The estimations of particle sizes by the two methods show good agreement.

The values 1·18 and 1·20 found for the densities of vaccinia and influenza viruses respectively are significantly higher than the densities of bacteria, which range from 1·05 to 1·15. This suggests a more compact structure for these viruses than that possessed by bacteria. The density of Staph. phage (Krueger) has been determined to be 1·25 (Elford, 1936), and the interesting question arises as to whether some of the smallest viruses and phages may not prove to be still somewhat more dense, and in this respect resemble protein substance in having a density 1·30–1·35.

The particle diameters determined by the centrifugal method used in the present study are compared in the following table with the corresponding figures found in the earlier ultrafiltration studies:

Virus.	Particle diameter in mμ.	
	Centrifugation.	Ultrafiltration.
Vaccinia	170–180	125–175
Influenza	87–99	80–120
Rous sarcoma	60–70	70–100

The results furnished by the two methods are seen to be in good agreement.

SUMMARY.

1. The particle diameter of vaccinia virus has been determined by means of centrifugation to be 170–180 mμ., that of influenza virus 87–99 mμ., and that of Rous sarcoma virus estimated to be 60–70 mμ.

2. Vaccinia virus is found to have a density of $1{\cdot}18 \pm 0{\cdot}02$ and influenza virus $1{\cdot}20 \pm 0{\cdot}05$.

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